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ErbB4 is a member of the signaling network composed of the EGF (epidermal growth factor) family of peptide hormones and the ErbB family of receptor tyrosine kinases. We have hypothesized that ErbB4 is a mammary-specific tumor suppressor. Indeed, we have preliminary data that indicates that overexpression of ErbB4 causes the EGF family hormones EGF and Neuregulin to inhibit DNA synthesis of a human mammary tumor cell line. Furthermore, we have constructed three constitutively active, ligand-independent ErbB4 mutants. We have published data indicating that these mutants do not malignantly transform the growth of cultured rodent fibroblasts. These data indicate that ErbB4 is not an oncogene. Moreover, we have preliminary data indicating that one of the three constitutively active ErbB4 mutants inhibits proliferation of several human mammary cell lines. Analyses of additional ErbB4 mutants suggest that this growth inhibition is dependent on ErbB4 kinase activity and on phosphorylation of tyrosine 1056 in the carboxyl-terminal region of the ErbB4 cytoplasmic domain.

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Table of Contents

Introduction

ErbB4 is a member of the signaling network composed of the EGF (epidermal growth factor) family of peptide hormones and the ErbB family of receptor tyrosine kinases. We have hypothesized that ErbB4 is a mammary-specific tumor suppressor. Indeed, we have preliminary data that indicate that overexpression of ErbB4 causes the EGF family hormones EGF and Neuregulin to inhibit DNA synthesis of a human mammary tumor cell line.

This IDEA award is partially supporting our efforts to elucidate the role of ErbB4 in mammary tumorigenesis. The five tasks enumerated in the approved Statement of Work are to (1) generate a constitutively-active ErbB4 mutant; (2) assess whether the constitutively-active ErbB4 mutant inhibits the proliferation of human mammary cell lines; (3) assess whether the constitutively-active ErbB4 mutant malignantly transforms the growth of human mammary cell lines; (4) generate ErbB4 double mutants in the context of the constitutively active mutant that are either deficient for kinase activity or contain mutations at tyrosine phosphorylation sites; (5) assess whether ErbB4 kinase activity or specific ErbB4 tyrosine phosphorylation sites are critical for coupling to biological activity.

Report Body

1. Generate a constitutively active ErbB4 mutant. The overall goals of the proposed research are to generate a constitutively active ErbB4 mutant and to use this mutant to probe ErbB4 function. We attempted to construct a constitutively active ErbB4 mutant by deleting the extracellular ligand-binding domain. However, we could not stably express this ErbB4 mutant. We attempted to construct a constitutively active ErbB4 mutant by replacing the transmembrane domain of human wild-type ErbB4 with the transmembrane domain of a constitutively active rat ErbB2/HER2/Neu mutant. However, we could not stably express this ErbB2-ErbB4 chimeric protein.

Our third attempt to generate a constitutively active ErbB4 mutant involved introducing single cysteine substitutions at five different locations in the ErbB4 extracellular juxtamembrane domain. Of these five ErbB4 mutants, three (Q646C, H647C, A648C) appear to be constitutively active for signaling; they exhibit greater kinase activity in the absence of ligand than does wild-type ErbB4 and they exhibit more tyrosine phosphorylation in the absence of ligand than does wild-type ErbB4 [1]. Thus, we have completed this task. These data were reported in the Annual Report submitted in July 2002. Thus, they are not shown here. However, they can be found in a reprint included in the appendix (Penington, *et al.*, 2002).

2. Assess whether the constitutively active ErbB4 mutant inhibits the proliferation of human mammary cell lines. Expression of ErbB4 and its ligands in rodent mammary glands peaks during a period of mammary gland differentiation late in pregnancy just prior to childbirth [2]. We have preliminary data that indicate that ErbB4 overexpression in the SKBR3 human mammary tumor cell line causes the EGF family hormones EGF and NRG1β to inhibit cellular DNA synthesis (Figure 1). This is consistent with published data from a competing laboratory demonstrating that ErbB4 expression in a panel of human mammary cell lines correlates with growth inhibition by NRG1β [3]. These published and unpublished data suggests that ErbB4 is coupled to growth arrest and that ErbB4 may be a tumor suppressor.

We have developed a simple assay for growth inhibition by the constitutively active ErbB4 mutants (Figure 2). We have infected cell lines of interest (human breast and prostate cell lines) with recombinant retroviruses that express the constitutively active ErbB4 mutants as well as a selectable marker (the neomycin resistance gene, which confers resistance to the antibiotic G418). We have also infected these cell lines of interest with control recombinant retroviruses that contain the neomycin resistance gene alone (vector control) or with retroviruses that contain the neomycin resistance gene along with either wild-type ErbB4 or a constitutively active ErbB2 mutant (ErbB2*). Following infection, we have selected for drug resistance. We divide the number of drug-resistant colonies by the volume of virus used in the infection to determine the viral titer for each combination of virus stock and cell. In parallel we determine the titer of each virus stock in the control mouse C127 fibroblast cell line (which has been shown to be nonresponsive to ErbB4 signaling [1]). Finally, we quantify growth inhibition by each virus by dividing the viral titer in the cell lines of interest by the viral titer in mouse C127 cells. This ratio will be reduced for those stocks that are growth inhibitory in the cell lines of interest (Figure 2).

Using this strategy, we have shown that the Q646C ErbB4 mutant inhibits formation of drug-resistant colonies in plastic dishes by the DU-145 and PC-3 human prostate tumor cell lines [4]. These data were reported in the Annual Report submitted in July 2002. Thus, they are not

shown here. However, they can be found in a reprint included in the appendix (Williams, *et al.*, 2003).

More recently, we have shown that the Q646C ErbB4 mutant inhibits formation of drug-resistant colonies in plastic dishes by the MCF-10A human mammary epithelial cell line and the MCF-7 and SKBR-3 human mammary tumor cell lines (Figure 3, Figure 4, and Figure 5). The Q646C ErbB4 mutant typically inhibits colony formation by 60-70% and this inhibition is statistically significant ($p \leq 0.05$ comparing ErbB4 Q646C to the vector control). However, the Q646C ErbB4 mutant does not inhibit colony formation by the MDA-MB-453 and T47D human mammary tumor cell lines (Figure 6).

Next, we wished to determine the effect of signaling by the Q646C ErbB4 mutant on MCF-10A cells. We speculated that ErbB4 signaling is coupled to growth arrest or to apoptosis in these cells. We distinguished between these two possibilities by modifying an assay that we had developed to demonstrate that the bovine papillomavirus E2 gene causes growth arrest in human cervical cancer cell lines [5]. We infected 1000 MCF-10A or C127 cells with 10000 colony-forming units (cfu) of the recombinant retrovirus that expresses wild-type ErbB4 or with an equal amount of the recombinant retrovirus that expresses the Q646C ErbB4 mutant. At the time of infection, we circled 20 isolated cells using a permanent marker. Four to seven days after infection we examined the marked cells to determine whether they remained present as single cells, whether they were absent, or whether they had formed a colony of cells. This enabled us to determine whether the Q646C ErbB4 mutant specifically couples to growth arrest (cell is present), apoptosis (cell is absent), or has no effect on cell behavior (cell forms a colony).

In multiple trials, expression of wild-type ErbB4 has no effect on colony formation by MCF-10A cells (Figure 7 and Figure 8). However, compared to mock infected MCF-10A cells, expression of the Q646C ErbB4 mutant inhibits colony formation by MCF-10A cells by more than 50% and increases growth arrest of MCF-10A cells by more than 100% (Figure 8). The inhibition of colony formation and increase in growth arrest by the Q646C ErbB4 mutant is statistically significant ($p<0.005$ in both cases – see Figure 8). In contrast, in C127 fibroblasts the Q646C ErbB4 does not significantly inhibit colony formation or increase growth arrest (Figure 8). Thus, constitutive ErbB4 signaling inhibits colony formation on plastic by coupling to growth arrest.

Finally, we attempted to generate stable cell lines following infection of a panel of human mammary cell lines (MCF-7, MCF-10A, SKBR-3) with the recombinant retroviruses that express the constitutively active ErbB4 mutants. We had hoped to assess whether the Q646C ErbB4 mutant or either of the other two constitutively active ErbB4 mutants affects mammary cell growth rates or saturation densities. Unfortunately, very few drug-resistant colonies arose following infection with the recombinant retrovirus that carries the Q646C ErbB4 mutant. This is not surprising given that the Q646C ErbB4 mutant is growth inhibitory. Furthermore, those colonies that did arise failed to ectopically express wild type ErbB4 (data not shown). This is not surprising given that expansion of cells infected by the retrovirus that carries the Q646C ErbB4 mutant would select for those cells that fail to express the Q646C ErbB4 mutant. In any event, not only have we been unable to assess the effects of ErbB4 signaling on MCF-7, MCF-10A and SKBR3 cells, we have also been unable to generate the cell lines necessary for the third task (below).

3. Assess whether the constitutively active ErbB4 mutant malignantly transforms the growth of human mammary cell lines. The constitutively active ErbB4 mutants do not

malignantly transform the growth of rodent fibroblast cell lines [1]. They do not induce growth in semi-solid medium in an assay for anchorage independence. They do not stimulate the growth rate or saturation density. Finally, they do not induce foci in an assay for loss of contact inhibition. These data were reported in the Annual Report submitted in July 2002. Thus, they are not shown here. However, they can be found in a reprint included in the appendix (Penington, *et al.*, 2002).

Because the constitutively active ErbB4 mutants fail to malignantly transform the growth of fibroblasts we believe it is unlikely that the constitutively active ErbB4 mutants will stimulate or increase anchorage-independent growth human mammary tumor cell lines. Furthermore, as we stated earlier, we are unable to stably express the Q646C ErbB4 mutant in human mammary cell lines. So, not only are we unable to pursue the experiments described in this task, there are no data that suggest that ErbB4 will malignantly transform the growth of human mammary cell lines. Thus, we believe that we have pursued this task to its conclusion, even though we have not been able to explicitly complete the task.

4. Generate ErbB4 double mutants in the context of the constitutively active mutant that are either deficient for kinase activity or contain mutations at tyrosine phosphorylation sites. We have constructed an ErbB4 mutant that is deficient for tyrosine kinase activity (K751M) and subcloned it into the background of the constitutively active ErbB4 Q646C mutant. This ErbB4 double mutant is designated Q646C Kin$^-$. We have also constructed an ErbB4 mutant that has all nine putative tyrosine phosphorylation sites (Y1022, Y1056, Y1150, Y1162, Y1188, Y1202, Y1242, Y1258, and Y1284) mutated to phenylalanine. This mutant is designated Chg9F. We have also generated a set of mutants that have all but one of the nine putative tyrosine phosphorylation sites mutated to phenylalanine. The critical mutant of this set is the mutant that retains the putative tyrosine phosphorylation site at amino acid 1056 but has had the remaining putative tyrosine phosphorylation sites mutated to phenylalanine. This mutant is designated Chg8F-Y1056. Both the Chg9F and Chg8F-Y1056 mutants have been subcloned into the background of the constitutively active ErbB4 Q646C mutants. These mutants are designated Q646C Chg9F and Q646C Chg8F-Y1056.

5. Using the ErbB4 double mutants, assess whether ErbB4 kinase activity or specific ErbB4 tyrosine phosphorylation sites are critical for coupling to biological activity. The ErbB4 Q646C Kin$^-$ mutant does not inhibit drug-resistant colony formation by the MCF-10A human mammary epithelial cell line or by the MCF-7 or SKBR-3 human mammary breast tumor cell lines (Figure 2, Figure 3, Figure 4, Figure 5). Thus, ErbB4 tyrosine kinase activity is required for the constitutively active Q646C ErbB4 mutant to couple to growth inhibition in these three cell lines.

Preliminary data indicate that the ErbB4 Q646C Chg9F mutant fails to inhibit drug-resistant colony formation by the MCF-10A and MCF-7 cell lines. In contrast, the ErbB4 Q646C Chg8F-Y1056 mutant inhibits drug-resistant colony formation by the MCF-10A and MCF-7 cell lines to the same extent as the ErbB4 Q646C mutant (Figure 9). Thus, phosphorylation of ErbB4 tyrosine 1056 appears to be necessary and possibly sufficient to couple ErbB4 to growth inhibition of human mammary cell lines.

Key Research Accomplishments

Task 1

- Generated a number of putative constitutively active ErbB4 mutants.
- Identified three ErbB4 mutants that exhibit ligand-independent tyrosine phosphorylation and increased tyrosine kinase activity.

Task 2

- Demonstrated that the constitutively active Q646C ErbB4 mutant inhibits drug-resistant colony formation by MCF-10A human mammary epithelial cells and by the SKBR-3 and MCF-7 human mammary tumor cell lines. Demonstrated that none of the constitutively active ErbB4 mutants inhibits drug-resistant colony formation by the MDA-MB-453 and T-47D human mammary tumor cell lines.
- Demonstrated that the constitutively active Q646C ErbB4 mutant inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines.
- Tried to generate a panel of human mammary (tumor) cell lines that express the constitutively active ErbB4 mutants. Demonstrated that the Q646C ErbB4 mutant is not expressed in infected MCF-10A, MCF-7 or SKBR-3 cell lines.

Task 3

- Demonstrated that the constitutively active ErbB4 mutants do not malignantly transform the growth of rodent fibroblast cell lines.
- For technical reasons described elsewhere in this report, we have been unable to pursue this task further.

Task 4

- Generated a kinase-deficient version of the constitutively active ErbB4 Q646C mutant.
- Generated versions of the constitutively active ErbB4 Q646C that lack all or all but one of the putative sites of ErbB4 tyrosine phosphorylation.

Task 5

- Demonstrated that a kinase-deficient version of the constitutively active ErbB4 Q646C mutant fails to inhibit drug-resistant colony formation by the MCF-10A, MCF-7, and SKBR-3 cell lines. Thus, demonstrated that kinase activity is required to couple the constitutively active ErbB4 Q646C mutant to growth inhibition in these cells.
- Tentatively demonstrated that the ErbB4 Q646C YChg9 mutant fails to inhibit drug-resistant colony formation by the MCF-10A and MCF-7 cell lines, whereas the ErbB4 Q646C YChg8-Y1056 mutant does inhibit drug-resistant colony formation by these same two cell lines. Thus, tentatively demonstrated that phosphorylation of ErbB4 tyrosine 1056 appears to be necessary and possibly sufficient to couple ErbB4 to growth inhibition of human mammary cell lines.

Reportable Outcomes

Meeting Abstracts Related to Project

Pitfield, S.E., E.E Williams, L.J. Trout, R.M. Gallo, I. Bryant, D.J. Penington and D.J. Riese II. "A Constitutively Active ErbB4 Mutant Inhibits Drug-Resistant Colony Formation By Human Breast and Prostate Cell Lines." Nineteenth Annual Meeting On Oncogenes, Hood College, Frederick, MD, June 2003.

Penington, D.J., E.E. Williams, S.S. Hobbs, J. Vanderpoel, R.M. Gallo, S. Slavik, E.M. Cameron, I. Bryant, A.T.D. Le, E.N. Blommel, S. Shukla, R.P. Hammer, V.J. Watts, and D.J. Riese II. "Multifaceted Approach to Study and to Target ErbB Family Receptor Signaling and Coupling to Biological Responses." Era of Hope Meeting, Orlando, FL, September 2002.

Williams, E.E., I. Bryant, S. Slavik, S. Shukla, J. Martin, D.J. Penington, and D.J. Riese II. "A Constitutively-Active ErbB4 Mutant Inhibits Colony Formation by Human Breast and Prostate Cell Lines." Era of Hope Meeting, Orlando, FL, September 2002.

Bryant, I., E.E. Williams, L.J. Trout, S. Pitfield, S. Shukla, J. Martin, R.M. Gallo, D.J. Penington, and D.J. Riese II. "A Constitutively Active ErbB4 Mutant Inhibits Colony Formation by Human Breast and Prostate Cell Lines." Tyrosine Phosphorylation and Signal Transduction Meeting, Salk Institute, La Jolla, CA, August 2002.

Williams, E.E. and D.J. Riese II. "A Constitutively Active ErbB4 Mutant Inhibits Colony Formation by Human Prostate Tumor Cell Lines." American Association of Colleges of Pharmacy Annual Meeting, Kansas City, MO, July 2002.

Byrant, I., S. Shukla, E.E. Williams, J. Martin, D.J. Penington, and D.J. Riese II. "A Constitutively Active ErbB4 Mutant Inhibits Colony Formation by Human Breast and Prostate Cell Lines." American Society for Microbiology Annual Meeting, Salt Lake City, UT, May 2002.

Penington, D.J., S. Shukla, I. Bryant, J. Vanderpoel, E.E. Williams, S.S. Hobbs, E.M. Cameron, A.T.D. Le, E. Blommel, C. Denson, S. Sajan, A. Morris, S. Slavik, G. Park, F. Cruz-Guilloty, R.P. Hammer, D. Beussman, and D.J. Riese II. "Multifaceted Approach to Study and to Target ErbB Family Receptor Signaling and Coupling to Biological Responses." Gordon Conference on Protein Phosphorylation and Second Messengers, June 2001.

Penington, D.J., I. Bryant, S. Shukla, E.E. Williams, G. Park, F. Cruz-Guilloty, and D.J. Riese II. "Construction and Analyses of Constitutively Active ErbB4 Mutants." Tyrosine Phosphorylation and Signal Transduction Meeting, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, May 2001.

Reportable Outcomes (continued)

Publications Related to Project

Penington, D.J., I. Bryant, and D.J. Riese II. "Constitutively Active ErbB4 and ErbB2 Mutants Exhibit Distinct Biological Activity." *Cell Growth Differentiation 13*: 247-256 (2002).

Williams, E.E., L.J. Trout, R.M. Gallo, S.E. Pitfield, I. Bryant, D.J. Penington, and D.J. Riese II. "A Constitutively-Active ErbB4 Mutant Inhibits Drug-Resistant Colony Formation by the DU-145 and PC-3 Human Prostate Tumor Cell Lines." *Cancer Letters* **192**: 67-74 (2003).

Bryant, I., S.E. Pitfield, R.M. Gallo, G. Park, D.J. Penington, and D.J. Riese II. "ErbB4 Kinase Activity and Phosphorylation of ErbB4 Tyrosine 1056 Couple A Constitutively-Active ErbB4 Mutant to Growth Inhibition in Human Mammary Cell Lines." Manuscript in Preparation.

Funded Grant Applications Related to Project

A grant application submitted to the USAMRMC PCRP for additional funding to support our efforts to analyze ErbB4 function in prostate cancer cells was selected for funding (DAMD17-02-1-0130, Dr. David J. Riese II, PI).

We were awarded an undergraduate research fellowship by the American Association of Colleges of Pharmacy to support our efforts to analyze ErbB4 function in prostate cancer cells (Mr. Eric Williams, PI; Dr. David J. Riese II, mentor).

We were awarded an undergraduate research fellowship by the American Society for Microbiology to support our efforts to analyze ErbB4 function in breast and prostate cancer cells (Ms. Ianthe Bryant, PI; Dr. David J. Riese II, mentor).

Degrees Earned Related to Project

Mr. Desi Penington wrote and successfully defended a master's degree thesis entitled "Construction and analysis of constitutively-active mutants of the ErbB4 receptor tyrosine kinase" that is based on the results of the studies described in Task 2. Mr. Penington was awarded an MS in August 2001.

Mr. Eric Williams was awarded a PharmD in May 2003. His PharmD project was entitled, "Role of ErbB4 Signaling in Prostate Tumorigenesis."

Conclusions

We have made significant progress on the proposed research plan. We have generated three constitutively active ErbB4 mutants. These mutants do not malignantly transform the growth of fibroblasts. Indeed, one of these mutants (Q646C) is coupled to growth arrest and inhibition of drug-resistant colony formation in human breast and prostate cell lines. These data suggest that ErbB4 may act as a tumor suppressor gene in the mammary and prostate glands. Growth inhibition by the ErbB4 Q646C mutant requires ErbB4 kinase activity and phosphorylation of ErbB4 at tyrosine 1056. Thus, we have adequately addressed all five tasks of the approved statement of work.

References

1. Penington, D.J., I. Bryant, and D.J. Riese II. "Constitutively Active ErbB4 and ErbB2 Mutants Exhibit Distinct Biological Activity." *Cell Growth Differentiation 13*: 247-256 (2002).
2. Sartor, C.I., H. Zhou, E. Kozlowska, K. Guttridge, E. Kawata, L. Caskey, J. Harrelson, N. Hynes, S. Ethier, B. Calvo, and H.S. Earp III. "HER4 Mediates Ligand-Dependent Antiproliferative and Differentiation Responses in Human Breast Cancer Cells." *Mol. Cell. Biol.* **21**: 4265-4275 (2001).
3. Schroeder, J.A., and D.C. Lee. "Dynamic expression and activation of ErbB receptors in the developing mouse mammary gland." *Cell Growth Diff.* **9**: 451-464 (1998).
4. Williams, E.E., L.J. Trout, R.M. Gallo, S.E. Pitfield, I. Bryant, D.J. Penington, and D.J. Riese II. "A Constitutively-Active ErbB4 Mutant Inhibits Drug-Resistant Colony Formation by the DU-145 and PC-3 Human Prostate Tumor Cell Lines." *Cancer Letters* **192**: 67-74 (2003).
5. Bryant, I., S.E. Pitfield, R.M. Gallo, G. Park, D.J. Penington, and D.J. Riese II. "ErbB4 Kinase Activity and Phosphorylation of ErbB4 Tyrosine 1056 Couple A Constitutively-Active ErbB4 Mutant to Growth Inhibition in Human Mammary Cell Lines." Manuscript in Preparation.
6. Hwang, E.-S., D.J. Riese II, J. Settleman, L.A. Nilson, J. Honig, S. Flynn, and D. DiMaio. "Inhibition of Cervical Carcinoma Cell Line Proliferation by the Introduction of a Bovine Papillomavirus Regulatory Gene." *J. Virology* **67**: 3720-3729 (1993).

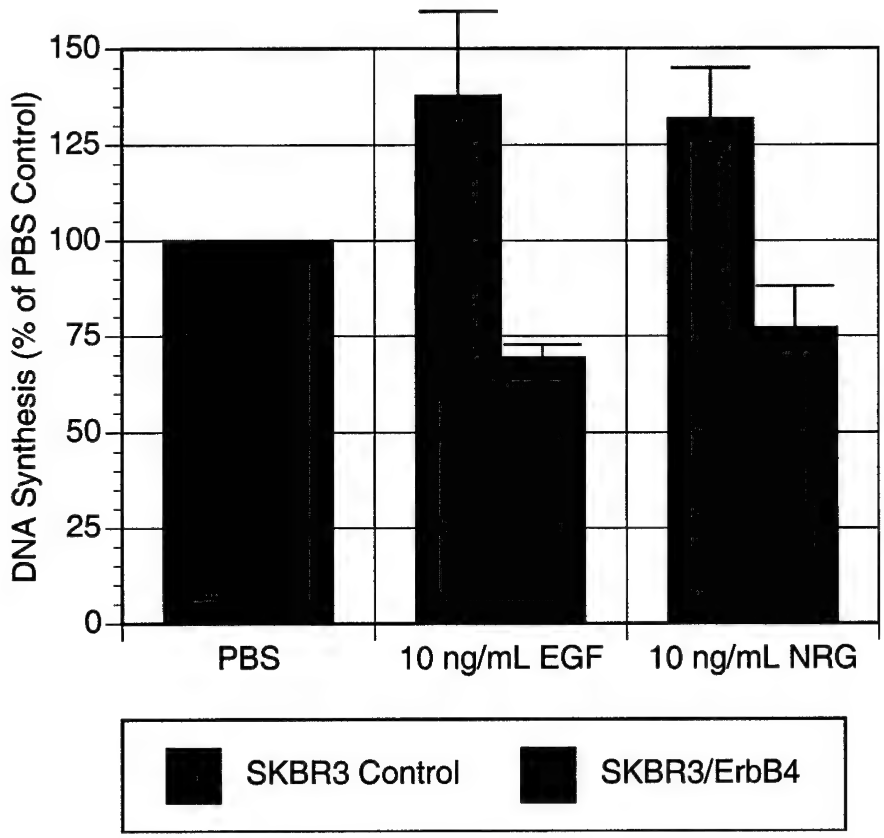
Appendices: List of Documents (27 pages total)

<u>*Figures (9 pages)*</u>

<u>*Journal Articles (18 pages)*</u>

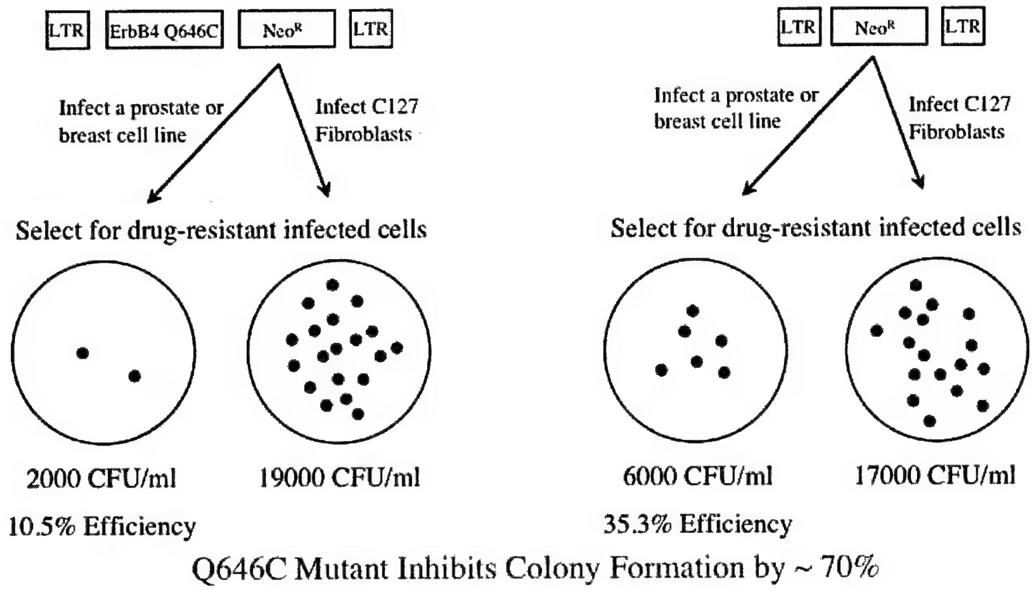
1. Penington, D.J., I. Bryant, and D.J. Riese II. "Constitutively Active ErbB4 and ErbB2 Mutants Exhibit Distinct Biological Activity." *Cell Growth Differentiation 13*: 247-256 (2002).
2. Williams, E.E., L.J. Trout, R.M. Gallo, S.E. Pitfield, I. Bryant, D.J. Penington, and D.J. Riese II. "A Constitutively-Active ErbB4 Mutant Inhibits Drug-Resistant Colony Formation by the DU-145 and PC-3 Human Prostate Tumor Cell Lines." *Cancer Letters* **192**: 67-74 (2003).

Figure 1. *Overexpression of ErbB4 in the SKBR3 human breast tumor cell line causes inhibition of DNA synthesis by EGF and NRG1β.*



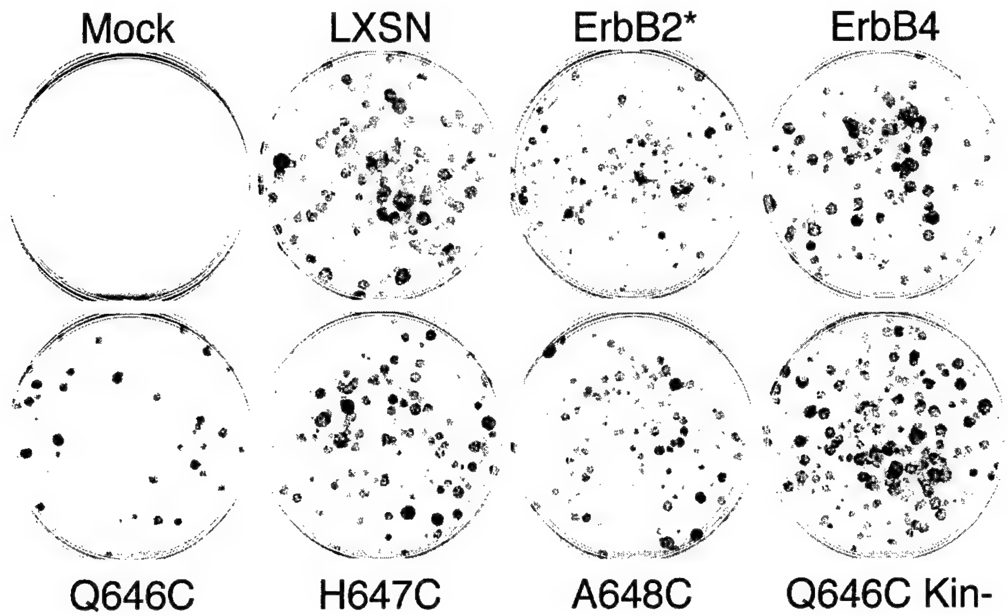
Control SKBR3 cells and an SKBR3 cells transfected with an ErbB4 expression vector (SKBR3/ErbB4) were starved overnight in serum free medium. They were then stimulated for 48 hours with 10 ng/mL EGF or NRG1β in fresh serum-free medium. DNA synthesis was assayed by measuring incorporation of ^{3}H-Thymidine. Results are expressed as a percentage of DNA synthesis observed following mock stimulation with phosphate buffered saline (PBS). Unpublished data.

Figure 2. *An assay for inhibition of drug-resistant colony formation can be used to identify growth inhibitory ErbB4 mutants.*



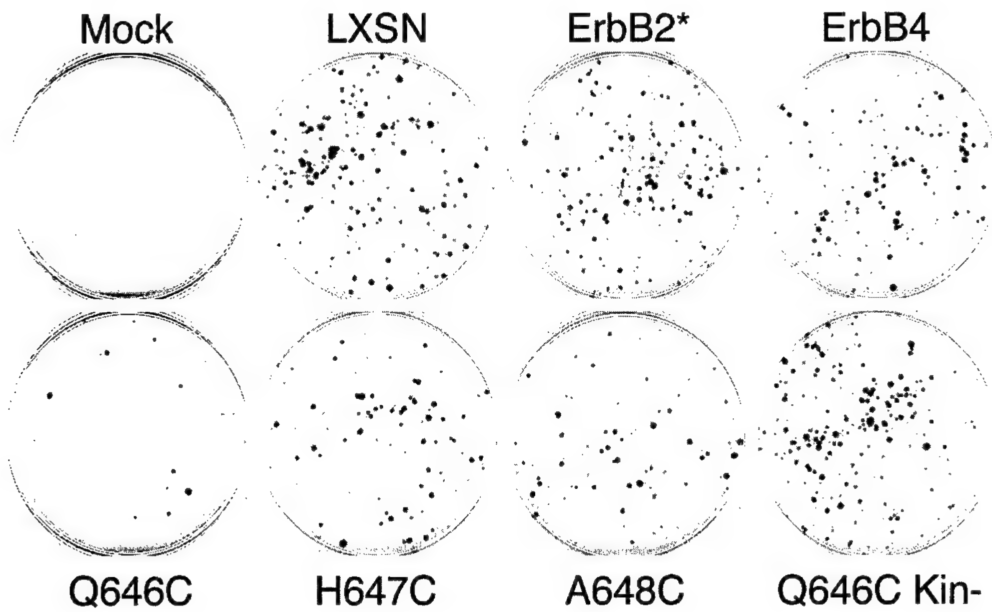
We have infected cell lines of interest (human breast and prostate cell lines) with recombinant retroviruses that express the constitutively active ErbB4 mutants as well as a selectable marker (the neomycin resistance gene, which confers resistance to the antibiotic G418). We have also infected these cell lines of interest with control recombinant retroviruses that contain the neomycin resistance gene alone (vector control) or with retroviruses that contain the neomycin resistance gene along with either wild-type ErbB4 or a constitutively active ErbB2 mutant (ErbB2*). Following infection, we have selected for drug resistance. We divide the number of drug-resistant colonies by the volume of virus used in the infection to determine the viral titer for each combination of virus stock and cell. In parallel we determine the titer of each virus stock in the control mouse C127 fibroblast cell line (which has been shown to be nonresponsive to ErbB4 signaling [1]). Finally, we quantify growth inhibition by each virus by dividing the viral titer in the cell lines of interest by the viral titer in mouse C127 cells. This ratio will be reduced for those stocks that are growth inhibitory in the cell lines of interest. In the example shown in the figure above, the Q646C mutant inhibits drug-resistant colony formation by approximately 70%. This value is calculated by dividing 10.5% by 35.3% and by subtracting this result from 100%. This strategy is described in detail in reference 4.

Figure 3. *The constitutively active Q646C ErbB4 mutant inhibits drug-resistant colony formation by the MCF10A human mammary epithelial cell line.*



MCF-10A human mammary epithelial cells were infected with recombinant retroviruses that express the neomycin resistance gene along with one of the three constitutively active ErbB4 mutants (Q646C, H647C, and A648C). As controls, MCF-10A cells were infected with a recombinant retrovirus that expresses only the neomycin resistance gene (LXSN), or the neomycin resistance gene together with either wild-type ErbB4 (ErbB4) or a constitutively active ErbB2 mutant (ErbB2*). MCF-7 cells were also mock infected or infected with a recombinant retrovirus that expresses the neomycin resistance gene along with a kinase-deficient, Q646C ErbB4 double mutant (Q646C Kin-). Following infection, cells were incubated in 600 μg/mL to select for infected, drug-resistant cells. After approximately 10 days of selection, colonies of drug-resistant cells were visualized by staining the tissue culture plates with Giemsa. The plates were rinsed, dried, and digitized using a UMAX Astra 2400S flatbed scanner set for a resolution of 300 dots per inch (dpi). This composite figure was assembled using Adobe Photoshop. This figure is taken from reference 5 and is representative of at least five independent sets of infections.

Figure 4. *The constitutively active Q646C ErbB4 mutant inhibits drug-resistant colony formation by the MCF7 human mammary tumor cell line.*



MCF-7 human mammary tumor cells were infected with recombinant retroviruses that express the neomycin resistance gene along with one of the three constitutively active ErbB4 mutants (Q646C, H647C, and A648C). As controls, MCF-7 cells were infected with a recombinant retrovirus that expresses only the neomycin resistance gene (LXSN), or the neomycin resistance gene together with either wild-type ErbB4 (ErbB4) or a constitutively active ErbB2 mutant (ErbB2*). MCF-7 cells were also mock infected or infected with a recombinant retrovirus that expresses the neomycin resistance gene along with a kinase-deficient, Q646C ErbB4 double mutant (Q646C Kin-). Following infection, cells were incubated in 600 μg/mL to select for infected, drug-resistant cells. After approximately 10 days of selection, colonies of drug-resistant cells were visualized by staining the tissue culture plates with Giemsa. The plates were rinsed, dried, and digitized using a UMAX Astra 2400S flatbed scanner set for a resolution of 300 dots per inch (dpi). This composite figure was assembled using Adobe Photoshop. This figure is taken from reference 5 and is representative of at least five independent sets of infections.

Figure 5. *The constitutively active Q646C ErbB4 mutant specifically inhibits drug-resistant colony formation by the MCF-10A human mammary epithelial cell line and the MCF-7 and SKBR3 human mammary tumor cell lines.*

	Viral titers				Colony formation efficiency		
Virus	Cell line				Ratios		
Stock	C127	MCF 7	MCF 10A	SKBR3	MCF 7/C127	MCF 10A/C127	SKBR3/C127
Vector	7.41E+05	6.89E+04	3.85E+04	5.64E+04	9.7% ± 1.4%	5.1% ± 0.8%	7.3% ± 1.3%
ErbB2*	3.22E+05	4.15E+04	1.63E+04	2.44E+04	15.2% ± 3.0%	5.1% ± 0.9%	7.0% ± 0.7%
ErbB4 WT	2.03E+05	2.49E+04	1.22E+04	1.51E+04	15.0% ± 3.2%	5.5% ± 0.5%	8.1% ± 1.7%
Q646C	7.18E+05	2.01E+04	1.43E+04	1.60E+04	* **3.0% ± 0.5%**	* **1.7% ± 0.2%**	* **2.2% ± 0.3%**
H647C	9.79E+05	8.01E+04	7.10E+04	5.21E+04	9.6% ± 1.4%	7.6% ± 0.8%	5.5% ± 0.9%
A648C	2.41E+05	2.91E+04	2.17E+04	1.75E+04	13.3% ± 2.0%	8.6% ± 0.7%	6.8% ± 0.9%
Q646C Kin-	4.96E+05	5.92E+04	3.20E+04	4.05E+04	14.7% ± 2.6%	5.6% ± 0.7%	9.1% ± 2.1%

* $p \leq 0.05$ (Comparing Q646C to Vector)

MCF-7, MCF-10A, and SKBR3 cells were infected with recombinant retroviruses that express the neomycin resistance gene along with one of the three constitutively active ErbB4 mutants (Q646C, H647C, and A648C). As controls, these cells were infected with a recombinant retrovirus that expresses only the neomycin resistance gene (Vector), or the neomycin resistance gene together with either wild-type ErbB4 (ErbB4 WT) or a constitutively active ErbB2 mutant (ErbB2*). These cells were also mock infected or infected with a recombinant retrovirus that expresses the neomycin resistance gene along with a kinase-deficient, Q646C ErbB4 double mutant (Q646C Kin$^-$). Following infection, cells were incubated in 600 μg/mL to select for infected, drug-resistant cells. In parallel, mouse C127 cells were infected and 1 mg/mL G418 was used to select for infected cells. After 10-20 days of selection, colonies of drug-resistant cells were visualized by staining the tissue culture plates with Giemsa.

The number of drug-resistant colonies was divided by the volume of virus used in the infection to determine the viral titer for each combination of virus stock and cell line. Colony formation efficiency for each combination of virus stock and cell line was calculated by dividing the viral titer in the cell lines of interest by the viral titer in mouse C127 cells. (The viral titer data for C127 cells can be found in Figure 5.) This ratio will be reduced for those stocks that are growth inhibitory in the cell lines of interest. In the figure shown above, the Q646C mutant inhibits drug-resistant colony formation by the MCF-7 cell lines by approximately 70%. This value is calculated by dividing 3.0% by 9.7% and by subtracting this result from 100%. This figure is taken from reference 5 and represents at least 5 independent sets of infections.

Figure 6. *The constitutively active Q646C ErbB4 mutant does not inhibit drug-resistant colony formation by the T47D and MDA-MB-453 human mammary tumor cell lines.*

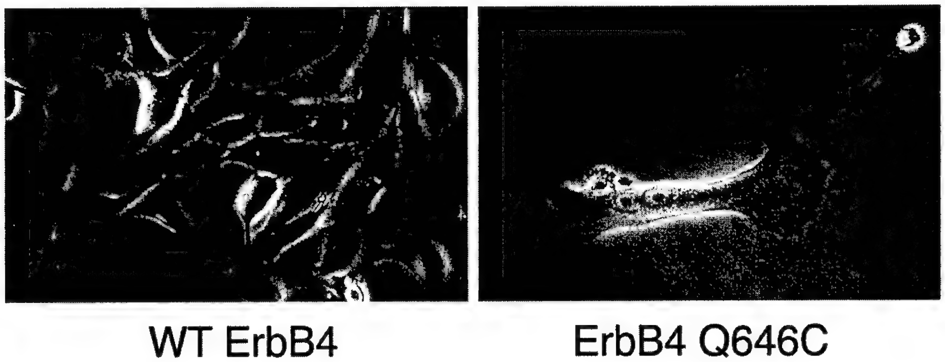
	Viral titers		Colony formation efficiency	
Virus	Cell line		Ratios	
Stock	MDA-MB453	T-47D	MDA-MB 453/C127	T-47D/C127
Vector	2.30E+04	1.71E+04	3.1% ± 0.7%	1.9% ± 0.6%
ErbB2*	1.80E+04	9.08E+03	6.7% ± 1.8%	2.5% ± 0.7%
ErbB4 WT	1.13E+04	4.36E+03	6.2% ± 1.4%	2.0% ± 0.4%
Q646C	1.72E+04	7.28E+03	***2.9% ± 1.0%**	****0.8% ± 0.2%**
H647C	3.17E+04	1.66E+04	3.6% ± 1.0%	1.5% ± 0.5%
A648C	7.95E+03	5.74E+03	4.0% ± 1.1%	2.2% ± 0.6%
Q646C Kin-	2.15E+04	1.18E+04	5.9% ± 2.0%	2.0% ± 0.6%

***p=0.848** ****p=0.096**
(Comparing Q646C to Vector)

MDA-MB-453 and T-47D cells were infected with recombinant retroviruses that express the neomycin resistance gene along with one of the three constitutively active ErbB4 mutants (Q646C, H647C, and A648C). As controls, these cells were infected with a recombinant retrovirus that expresses only the neomycin resistance gene (LXSN), or the neomycin resistance gene together with either wild-type ErbB4 (ErbB4) or a constitutively active ErbB2 mutant. These cells were also mock infected or infected with a recombinant retrovirus that expresses the neomycin resistance gene along with a kinase-deficient, Q646C ErbB4 double mutant. Following infection, cells were incubated in 600 μg/mL to select for infected, drug-resistant cells. In parallel, mouse C127 cells were infected and 1 mg/mL G418 was used to select for infected cells. After 10-20 days of selection, colonies of drug-resistant cells were visualized by staining the tissue culture plates with Giemsa.

The number of drug-resistant colonies was divided by the volume of virus used in the infection to determine the viral titer for each combination of virus stock and cell line. Colony formation efficiency for each combination of virus stock and cell line was calculated by dividing the viral titer in the cell lines of interest by the viral titer in mouse C127 cells. This ratio will be reduced for those stocks that are growth inhibitory in the cell lines of interest. However, as shown in the figure shown above, the Q646C mutant fails to inhibit drug-resistant colony formation by the MDA-MB-453 and T-47D cell lines. This figure is taken from reference 5 and represents at least five independent sets of infections.

Figure 7. *The constitutively active Q646C ErbB4 mutant causes growth arrest of MCF-10A human mammary epithelial cells.*



We infected 1000 MCF-10A or C127 cells with 10000 colony-forming units (cfu) of the recombinant retrovirus that expresses wild-type ErbB4 or with an equal amount of the recombinant retrovirus that expresses the Q646C ErbB4 mutant. At the time of infection, we circled 20 isolated cells using a permanent marker. Four to seven days after infection we examined the marked cells to determine whether they remained present as single cells, whether they were absent, or whether they had formed a colony of cells. Photomicrographs were taken of representative fields. This figure is taken from reference 5 and is representative of the results of three independent sets of experiments.

Figure 8. *The constitutively active Q646C ErbB4 mutant causes growth arrest, but not apoptosis, of MCF-10A human mammary epithelial cells.*

		Cell Fate Following Infection		
Cell Line	Virus	Present	Absent	Colony
C127	Mock	3.0	0.0	17.0
	ErbB4	5.0	0.5	14.5
	ErbB4 Q646C	5.5	0.0	14.5
	ErbB4 vs Q646C	**p=0.841**		**p=0.978**
MCF 10A	Mock	6.0	2.3	11.8
	ErbB4	4.5	2.8	12.8
	ErbB4 Q646C	13.3	1.3	5.5
	ErbB4 vs Q646C	**p=0.003**		**p=0.001**

We infected 1000 MCF-10A or C127 cells with 10000 colony-forming units (cfu) of the recombinant retrovirus that expresses wild-type ErbB4 or with an equal amount of the recombinant retrovirus that expresses the Q646C ErbB4 mutant. At the time of infection, we circled 20 isolated cells using a permanent marker. Four to seven days after infection we examined the marked cells to determine whether they remained present as single cells, whether they were absent, or whether they had formed a colony of cells. The results were tabulated and averages from three independent sets of infections were calculated. This enabled us to determine whether the Q646C ErbB4 mutant specifically couples to growth arrest (cell is present), apoptosis (cell is absent), or has no effect on cell behavior (cell forms a colony). This figure is taken from reference 5.

Figure 9. *Phosphorylation of tyrosine 1056 is necessary and possibly sufficient to couple the ErbB4 Q646C mutant to inhibition of drug-resistant colony formation by MCF-10A and MCF-7 cells.*

Virus Stock	Viral Titers, Cell Line: C127	Viral Titers, Cell Line: MCF-10A	Colony Formation Efficiency Ratios MCF-10A/C127
Vector	5.43E+05	1.13E+04	2.01%
ErbB4 WT	7.37E+05	1.63E+04	2.16%
Q646C	1.24E+06	5.50E+03	0.43%
Q646C Chg8F-Y1056	2.98E+06	9.15E+03	0.22%
Q646C Chg9F	1.93E+04	5.85E+02	3.24%

MCF-10A cells were infected with recombinant retroviruses that express the neomycin resistance gene along with one of the three following ErbB4 mutants: Q646C, Q646C Chg8F-Y1056, or Q646C Chg9F. As controls, these cells were infected with a recombinant retrovirus that expresses only the neomycin resistance gene (LXSN), or the neomycin resistance gene together with wild-type ErbB4 (ErbB4 WT). These cells were also mock infected. Following infection, cells were incubated in 600 μg/mL to select for infected, drug-resistant cells. In parallel, mouse C127 cells were infected and 1 mg/mL G418 was used to select for infected cells. After 10-20 days of selection, colonies of drug-resistant cells were visualized by staining the tissue culture plates with Giemsa.

The number of drug-resistant colonies was divided by the volume of virus used in the infection to determine the viral titer for each combination of virus stock and cell line. Colony formation efficiency for each virus stock was calculated by dividing the viral titer in the MCF-10A cells by the viral titer in mouse C127 cells. This ratio will be reduced for those stocks that are growth inhibitory in the cell lines of interest. In the figure shown above, the Q646C mutant inhibits drug-resistant colony formation by the MCF-7 cell lines by approximately 80%. This value is calculated by dividing 0.43% by 2.01% and by subtracting this result from 100%. This figure is taken from reference 5 and represents two independent sets of infections.

Constitutively Active ErbB4 and ErbB2 Mutants Exhibit Distinct Biological Activities[1]

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Abstract

ErbB4 is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, which includes EGFR/ErbB1, ErbB2/HER2/Neu, and ErbB3/HER3. These receptors play important roles both in normal development and in neoplasia. For example, deregulated signaling by ErbB1 and ErbB2 is observed in many human malignancies. In contrast, the roles that ErbB4 plays in tumorigenesis and normal biological processes have not been clearly defined. To identify the biological responses that are coupled to ErbB4, we have constructed three constitutively active ErbB4 mutants. Unlike a constitutively active ErbB2 mutant, the ErbB4 mutants are not coupled to increased cell proliferation, loss of contact inhibition, or anchorage independence in a rodent fibroblast cell line. This suggests that ErbB2 and ErbB4 may play distinct roles in tumorigenesis *in vivo*.

Introduction

ErbB4 (HER4/p180^{erbB4}) is a member of the EGFR[3] (EGFR/ErbB) family of receptor tyrosine kinases. These receptors play important roles in the embryonic development of heart, lung, and nervous tissues (1–4), and they have been implicated in the progression of metastatic disease. For example, EGFR/ErbB1 is overexpressed, amplified, or mutated in a number of human malignancies including breast, ovary, prostate, and lung cancers (5–7). ErbB2 overexpression correlates with tumor aggressiveness and poor prognosis in node-positive breast cancer patients (reviewed in Ref. 8). Finally, ErbB3 overexpression is observed in a subset of human mammary and gastric cancers (9, 10).

Some reports indicate that increased ErbB4 expression or signaling is associated with tumorigenesis. ErbB4 overexpression has been observed in a variety of cancers, including tumors of the thyroid, breast, and gastrointestinal tract (11–14). However, the prognostic significance of ErbB4 expression in tumors may also depend on which ErbB family members are coexpressed with ErbB4. In the case of childhood medulloblastoma (one of the most common solid tumors of childhood), patients with tumors overexpressing both ErbB2 and ErbB4 have a significantly worse prognosis than patients with tumors that overexpress either receptor alone (15).

Other reports indicate that increased ErbB4 expression or signaling correlates with tumor cell differentiation and reduced aggressiveness. ErbB4 overexpression in breast tumors is associated with progesterone receptor and estrogen receptor expression and a more favorable prognosis (16–17). In contrast, ErbB2 overexpression varies inversely with progesterone receptor and estrogen receptor levels and indicates tumors that are more likely to be metastatic and fatal (18). In one survey of common solid human cancers, the loss of ErbB4 expression is seen in a significant percentage of breast, prostate, and head and neck malignancies (19). These findings raise the intriguing possibility that ErbB4 is unique to the ErbB family of receptors in that ErbB4 expression and signaling may couple to reduced tumorigenesis or tumor cell proliferation. However, in the face of the conflicting evidence we have summarized here, it remains unclear what general or specific roles ErbB4 plays in differentiation, tumor suppression, or proliferation.

Efforts to elucidate ErbB4 function have been hampered by many factors. There are no known agonists or antagonists specific to the ErbB4 receptor. All of the peptide hormones of the EGF family that are capable of binding ErbB4 also bind at least one other ErbB family member. For example, epiregulin and betacellulin bind and activate both ErbB1 and ErbB4 (20, 21). Furthermore, ligands that do not bind an ErbB family receptor can still activate signaling by that receptor in *trans* through ligand-induced receptor heterodimerization (reviewed in Refs. 22, 23). For example, EGF stimulates ErbB2 tyrosine phosphorylation when ErbB2 is coexpressed with ErbB1, whereas EGF will not stimulate ErbB2 tyrosine phosphorylation in the absence of ErbB1 (24). Consequently, ligands that bind and directly activate ErbB4 (neuregulin, betacellulin, and epiregulin) also stimulate ErbB1, ErbB2, and ErbB3 signaling (Refs. 20, 21, 25, 26; reviewed in Refs. 22, 23). Therefore, in most contexts it is virtually impossible to use an EGF family hormone to study the functional consequences of ErbB4 signaling.

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; cfu, colony-forming unit(s); FR3T3, Fischer rat 3T3; LMP, low melting point; LTR, long terminal repeat.

To study ErbB4 function, we have opted to generate ErbB4 mutants that contain a cysteine substitution in the extracellular domain. This is predicted to result in constitutively dimerized and constitutively active ErbB4 mutants. Introducing cysteine residues to form covalently linked, dimeric, constitutively active receptor tyrosine kinases is not novel. This strategy has been used to generate dimeric, constitutively active mutants of EGFR/ErbB1 and ErbB2 (27, 28). Cysteine substitutions also lead to constitutively active mutants of the fibroblast growth factor receptors 2 and 3 (29, 30).

Here we report the generation and characterization of three constitutively active ErbB4 mutants. These mutants were generated through the introduction of a cysteine residue in the extracellular region of ErbB4. These mutants exhibit increased ligand-independent ErbB4 tyrosine phosphorylation, dimerization, and kinase activity. However, these constitutively active ErbB4 mutants do not induce increased proliferation, loss of contact inhibition, or anchorage-independent growth in FR3T3 fibroblasts. In contrast, a constitutively active ErbB2 mutant does induce increased proliferation, loss of contact inhibition, and anchorage-independent growth in FR3T3 fibroblasts. These results suggest that ErbB4 and ErbB2 couple to different signaling pathways and biological responses. These results also suggest that ErbB4 and ErbB2 may play distinct roles in tumorigenesis *in vivo*.

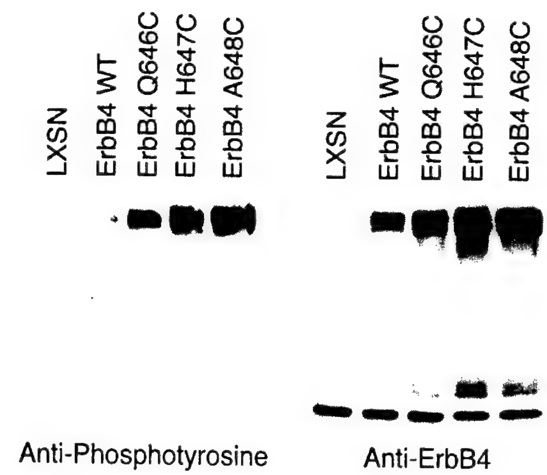

Fig. 1. ErbB4 mutants are constitutively tyrosine phosphorylated. ErbB4 expression and tyrosine phosphorylation were assayed in PA317 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants. Cells infected with the LXSN recombinant retrovirus vector control served as the negative control. Lysates were prepared from each of the cell lines, and ErbB4 was immunoprecipitated from 1000 μg of each lysate. Samples were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (*left panel*). The blot was then stripped and probed with an anti-ErbB4 rabbit polyclonal antibody (*right panel*). The band at the *top* of the blots represents ErbB4.

Results

ErbB4 Mutants Are Constitutively Tyrosine Phosphorylated. We substituted a single cysteine for amino acids Pro-645, Gln-646, His-647, Ala-648, and Arg-649 in the juxtamembrane region of the ErbB4 extracellular domain. These ErbB4 mutants (P645C, Q646C, H647C, A648C, and R649C) were generated in the context of the pLXSN-ErbB4 recombinant retroviral expression vector (26). Because these cysteine substitutions might cause inappropriate protein folding and decreased protein stability, we assayed the ErbB4 mutants for stable expression. We transfected the recombinant retroviral vectors containing the ErbB4 mutant constructs into the Ψ2 ecotropic retrovirus packaging cell line, selected for stable transformants, and generated pooled cell lines. We harvested low-titer ecotropic retrovirus stocks from these cell lines, and we analyzed the expression and tyrosine phosphorylation of the ErbB4 mutants in these cell lines. Three ErbB4 mutants (Q646C, H647C, and A648C) exhibit abundant expression and ligand-independent tyrosine phosphorylation (data not shown). However, the R649C ErbB4 mutant is not efficiently expressed, and the P645C mutant does not display ligand-independent tyrosine phosphorylation (data not shown).

Previous studies indicate that transfection and subsequent overexpression of ErbB family receptors lead to ligand-independent receptor tyrosine phosphorylation (31–33). Consequently, we were concerned that the ligand-independent phosphorylation of the Q646C, H647C, and A648C ErbB4 mutants in the transfected Ψ2 cells was a consequence of overexpression. Therefore, we infected the PA317 amphotropic retrovirus packaging cell line with the ErbB4 mutant recombinant ecotropic retroviruses at low multiplicities of infection (<0.1), selected for infected cells, and generated pooled cell lines. Because these cell lines were generated by infection at low multiplicities of infection, it is likely that each cell contains only one or two copies of the ErbB4 expression construct. This reduces the likelihood of ErbB4 overexpression in these cell lines.

We analyzed ErbB4 expression and tyrosine phosphorylation in the PA317 cell lines by anti-ErbB4 immunoprecipitation and either anti-ErbB4 (Fig. 1, *right panel*) or anti-phosphotyrosine (Fig. 1, *left panel*) immunoblotting. As expected, cells infected with the LXSN vector control retrovirus do not exhibit ErbB4 expression (Fig. 1, *right panel*) or tyrosine phosphorylation (Fig. 1, *left panel*). Cells infected with the wild-type or mutant ErbB4 retroviruses exhibit ErbB4 expression (Fig. 1, *right panel*). However, cells infected with the mutant ErbB4 retroviruses exhibit abundant ErbB4 tyrosine phosphorylation, whereas cells infected with the wild-type ErbB4 retrovirus exhibit minimal ErbB4 tyrosine phosphorylation (Fig. 1, *left panel*).

Quantification of the chemilumigrams shown in Fig. 1 suggests that the expression levels of the three ErbB4 mutants is less than three times greater than the amount of wild-type ErbB4 expression (Table 1). In contrast, the amounts of tyrosine phosphorylation of the three ErbB4 mutants appear to be much greater than the amount of wild-type ErbB4 tyrosine phosphorylation. Moreover, the ratios of ErbB4 tyrosine phosphorylation to ErbB4 expression for the three ErbB4 mutants appear to be at least four times greater than the ratio for wild-type ErbB4. These data suggest that the three ErbB4 mutants exhibit greater amounts of tyrosine phosphorylation on a per-molecule basis than does wild-type ErbB4. Consequently, these data indicate that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling.

Table 1 The Q646C, H647C, and A648C ErbB4 mutants exhibit increased normalized tyrosine phosphorylation

Cell line	ErbB4 tyrosine phosphorylation	ErbB4 expression	Ratio
Wild-type ErbB4	210000	1800000	0.12
ErbB4 Q646C	1900000	3300000	0.58
ErbB4 H647C	2900000	4700000	0.62
ErbB4 A648C	4000000	4500000	0.89

ErbB4 Mutants Have Increased *in Vitro* Kinase Activity. Next, we assessed whether the increased tyrosine phosphorylation of the three ErbB4 mutants correlates with increased kinase activity. Equal amounts of the same lysates used for the experiments described in Fig. 1 were immunoprecipitated with an anti-ErbB4 polyclonal antibody. Kinase reactions were performed on the immunoprecipitates in the presence of [γ-^{32}P]ATP. The reaction products were resolved by SDS-PAGE on a 7.5% acrylamide gel. The gel was dried, and the reaction products were visualized by autoradiography.

In Fig. 2, we show that PA317 cells infected with the LXSN vector control retrovirus lack detectable ErbB4 kinase activity. Moreover, PA317 cells that express the three constitutively active ErbB4 mutants exhibit greater ErbB4 tyrosine kinase activity than cells that express wild-type ErbB4. Quantification of the bands on the autoradiogram indicates that the Q646C and H647C ErbB4 mutants exhibit approximately five times more kinase activity than does wild-type ErbB4, whereas the A648C ErbB4 mutant exhibits approximately nine times more kinase activity than does wild-type ErbB4. Given that the expression of the ErbB4 mutants (in these same lysates) is somewhat greater than the expression of wild-type ErbB4 (Fig. 1 and Table 1), it appears that the intrinsic kinase activity of the three ErbB4 mutants is three to four times greater than the intrinsic kinase activity of wild-type ErbB4.

Constitutively Active ErbB4 Mutants Do Not Induce a Loss of Contact Inhibition. Once we determined that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling, we performed experiments using these mutants to identify the biological events coupled to ErbB4 signaling. A common assay for genes that encode growth control or signaling proteins involves introducing the gene into an established rodent fibroblast cell line and assaying for foci of piled-up cells. These foci indicate a loss of contact inhibition, a common attribute of malignant cells. Thus, this gene transfer assay is commonly used to identify genes that encode proteins that are coupled to malignant growth transformation.

Conflicting results have been obtained from assays for growth transformation by ErbB4. Transfection and consequent overexpression of ErbB4 induces foci (loss of contact inhibition) in NIH 3T3 clone 7 cells in the absence of ligand. Moreover, in these cells focus formation was stimulated by the ErbB4 ligand neuregulin 2β. In contrast, NIH 3T3 clone 7d cells (which lack EGFR expression) transfected with wild-type ErbB4 did not form foci in the presence or absence of neuregulin 1β; however, ErbB4 cotransfected with EGFR/ErbB1 or ErbB2 does induce foci in these cells (32, 33). One

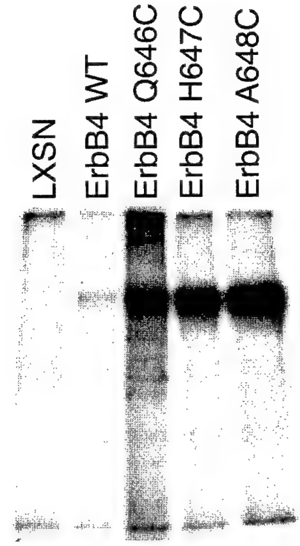

Fig. 2. Q646C, H647C, and A648C mutants exhibit increased *in vitro* kinase activity. Equal amounts of protein lysates (1000 μg) from PA317 cells that stably express wild-type ErbB4 or the ErbB4 mutants (Q646C, H647C, and A648C) were immunoprecipitated with an anti-ErbB4 rabbit polyclonal antibody. Lysates from PA317 cells that express the LXSN vector served as the negative control. Kinase reactions were performed on the immunoprecipitates in the presence of [γ-^{32}P]ATP. The products were resolved by SDS-PAGE. The gel was dried overnight and exposed to X-ray film for ~20 h to visualize the products of the kinase reactions.

possible explanation is that ErbB4 lacks intrinsic transforming activity but does permit EGFR/ErbB1 or ErbB2 signaling and coupling to growth transformation in the presence of an ErbB4 ligand.

To test whether ErbB4 signaling is sufficient to transform the growth of cultured rodent fibroblasts, FR3T3 fibroblasts were infected with 200 cfu of the ErbB4 mutant recombinant ecotropic retrovirus stocks and assayed for focus formation. Cells infected with 200 cfu of the LXSN vector control recombinant ecotropic retrovirus and with 200 cfu of the wild-type ErbB4 recombinant ecotropic retrovirus served as negative controls. Cells infected with 200 cfu of the constitutively active (V664E transmembrane domain) mutant ErbB2* retrovirus served as a positive control.

FR3T3 cells infected with the ErbB2* retrovirus had formed foci within 9 days after infection, whereas cells infected with the vector control retrovirus had not (Fig. 3). Furthermore, cells infected with the wild-type or mutant ErbB4 retroviruses had not formed foci within 9 days after infection. Within 18 days after infection, the foci arising from FR3T3 cells infected with the ErbB2* retrovirus had completely covered the surface of the tissue culture plate and had began to detach from the surface of the plate (data not shown). Within 18 days after infection, FR3T3 cells infected with the mutant ErbB4 retroviruses had formed relatively high-density clumps (data not shown). These high-density clumps did not exhibit the overlapping cell processes characteristic of foci (data not shown). The cells comprising these clumps were cloned and expanded into cell lines, as were cells from less dense regions of the cell monolayers. The cells from the clumps are morphologically indistinguishable from cells derived from the

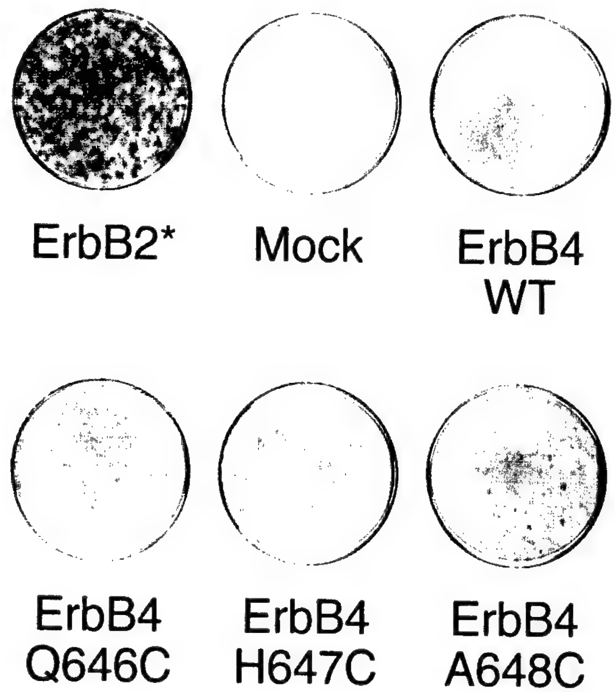

Fig. 3. Constitutively active ErbB4 receptors do not induce a loss of contact inhibition. FR3T3 fibroblasts infected with the LXSN (vector control) retrovirus, the wild-type ErbB4 retrovirus, the constitutively active ErbB2* retrovirus, or the constitutively active ErbB4 mutant retroviruses were assayed for loss of contact inhibition (focus formation).

less dense regions of the plates and are morphologically indistinguishable from cells that express wild-type ErbB4 or cells infected with the vector control retrovirus (data not shown). Again, this suggests that the constitutively active ErbB4 mutants do not transform the growth of FR3T3 fibroblasts.

We were concerned that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts might be specific to this cell type. Consequently, we performed similar experiments with mouse C127 fibroblasts. Infection with the ErbB2* retrovirus resulted in numerous foci, whereas infection with the constitutively active ErbB4 mutant retroviruses did not (data not shown). Thus, again, whereas the constitutively active ErbB2* mutant readily induces foci in fibroblasts, the constitutively active ErbB4 mutants do not. This suggests that ErbB2 and ErbB4 couple to distinct cellular signaling pathways and biological events.

Constitutively Active ErbB4 Mutants Do Not Induce Anchorage-independent Growth. Next, we assayed FR3T3 cells that express the constitutively active ErbB4 mutants for growth while suspended in semisolid medium. Because anchorage-independent growth is another characteristic attribute of tumor cells *in vivo*, this assay is another way to determine whether ErbB4 signaling is coupled to malignant growth transformation.

FR3T3 cells were infected with the ErbB4 mutant recombinant ecotropic retroviruses at a low multiplicity of infection, and infected cells were selected using G418. Drug-resistant colonies of cells were pooled and expanded into cell lines. Control cell lines were generated through infection of FR3T3 cells with the wild-type ErbB4 retrovirus, the constitutively active ErbB2 retrovirus, and with the LXSN vector control retrovirus. These cell lines were seeded at a density of 2×10^4 cells/ml in 60-mm dishes in semisolid medium containing 0.3% LMP-agarose. Fresh medium containing LMP-agarose was added every 3 days. Photographs were taken of representative fields after 10 days.

FR3T3 cells that express the constitutively active ErbB2* mutant exhibit anchorage-independent growth (Fig. 4). In contrast, cells that were infected with the LXSN recombinant retroviral vector control and cells that express wild-type ErbB4 or the ErbB4 mutants do not exhibit anchorage-independent growth. The results of this assay are consistent with the results of the focus formation assay; both assays indicate that ErbB4 signaling is distinct from ErbB2 signaling in that ErbB4 signaling is not coupled to malignant growth transformation in FR3T3 fibroblasts.

Constitutively Active ErbB4 Mutants Do Not Increase the Growth Rate or Saturation Density. Another characteristic of malignantly transformed fibroblasts is that their growth rates and saturation densities are higher than those of their nontransformed counterparts. Indeed, constitutive ErbB2 signaling is coupled to increased growth rates (reviewed in Ref. 8). Thus, we assessed whether the constitutively active ErbB4 mutants affected the growth rate or saturation density of FR3T3 fibroblasts. The FR3T3 cell lines described earlier were seeded in 60-mm dishes at a density of 2×10^4 cells/dish (700 cells/cm^2). Cells were incubated for 10 days to permit proliferation. During this period, cells were counted every 24 h.

The growth rate of the cells that express ErbB2* is slightly greater than the growth rates of the other cell lines (Fig. 5). Note that the growth rates of the cells that express the constitutively active ErbB4 mutants are indistinguishable from the growth rates of cell lines that express wild-type ErbB4 or the vector control. The growth curves in Fig. 5 were used to determine the saturation densities for the six cell lines (Table 2). Note that the saturation density of the cell line that expresses ErbB2* is higher than the saturation densities of the other cell lines. Moreover, the saturation densities of the cell lines that express the ErbB4 mutants are not markedly higher than the saturation densities of the vector control cell line or the cell line that expresses wild-type ErbB4. Once again, these data suggest that constitutive ErbB4 signaling is not coupled to malignant growth transformation in fibroblasts. Thus, the signaling pathways and biological responses that are coupled to ErbB4 are distinct from those that are coupled to ErbB2.

The Constitutively Active ErbB4 Mutants Are Expressed and Are Constitutively Tyrosine Phosphorylated in FR3T3 Cells. We were concerned that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts might be attributable to the absence of ErbB4 expression or constitutive tyrosine phosphorylation in these cells. In parallel with the infections described in Fig. 3, we infected FR3T3 cells with 200 cfu of the constitutively active mutant ErbB4 recombinant retroviruses

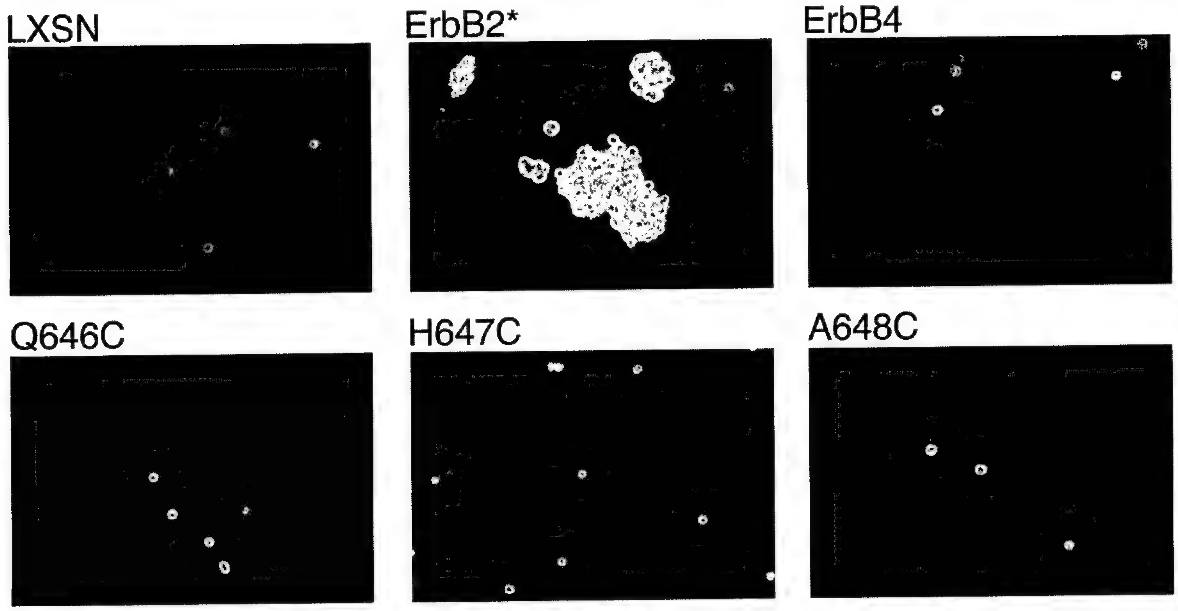

Fig. 4. Constitutively active ErbB4 receptors do not induce growth in semisolid medium. FR3T3 cells that stably express the LXSN vector control, the constitutively active ErbB2 mutant (ErbB2*), wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were seeded in semisolid medium at a density of 2×10^4 cells/ml in 60-mm dishes. The cells were incubated for 10 days, after which images were recorded by photomicroscopy. Images shown are representative of those obtained in three independent experiments.

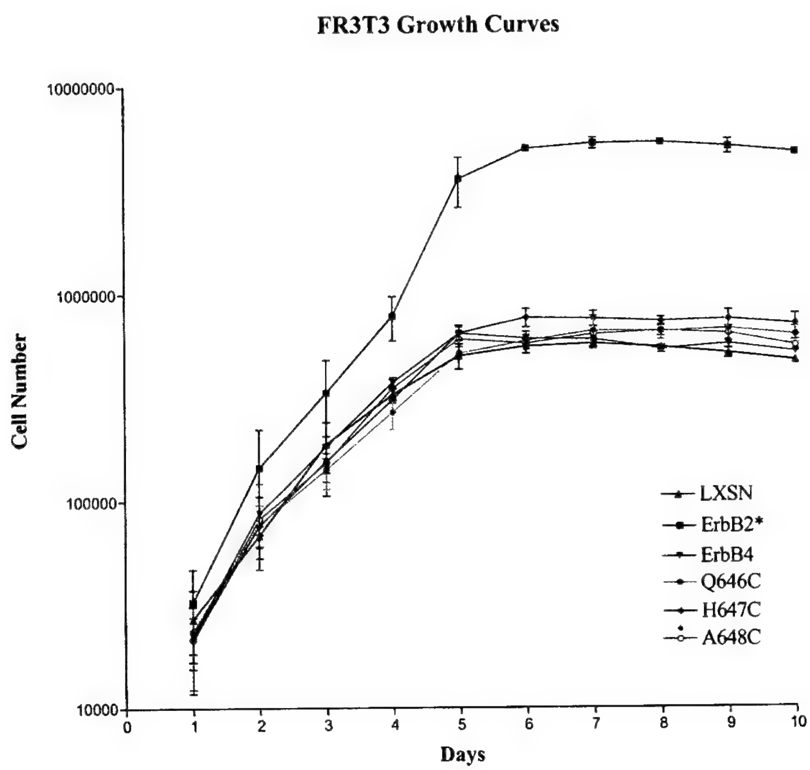

Fig. 5. Constitutively active ErbB4 mutants do not increase the growth rate of FR3T3 fibroblasts. FR3T3 cells that express the LXSN vector control, the constitutively active ErbB2* mutant, wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were plated at a density of 2×10^4 cells in 60-mm dishes (700 cells/cm^2) and were incubated for 1–10 days. Cells were counted daily to assess growth rates and saturation densities. The means for three independent experiments; *bars,* SE.

and selected for stable infection using G418. As controls, we also infected FR3T3 cells with 200 cfu of the vector control retrovirus, 200 cfu of the ErbB2* retrovirus, and with 200 cfu of the wild-type ErbB4 retrovirus. Drug-resistant colonies were pooled and expanded into stable cell lines. The cell lines were starved of serum in the presence of 500 μM Na_3VO_4 (34) to decrease the background level of tyrosine phosphorylation and to increase the phosphorylation of the

Table 2 Constitutively active ErbB4 mutants do not increase the saturation density of FR3T3 fibroblasts

Saturation Densities	
LXSN	$5.8 \pm 0.3 \times 10^5$
ErbB2*	$5.4 \pm 0.1 \times 10^6$
ErbB4	$6.1 \pm 0.5 \times 10^5$
Q646C	$6.6 \pm 0.6 \times 10^5$
H647C	$7.6 \pm 0.7 \times 10^5$
A648C	$6.6 \pm 0.4 \times 10^5$

constitutively active ErbB4 mutants. We prepared lysates and analyzed ErbB4 expression and tyrosine phosphorylation by precipitation with an anti-ErbB4 antibody and sequential anti-phosphotyrosine and anti-ErbB4 immunoblotting.

In Fig. 6, *lower panel,* we show that ErbB4 expression is detectable in the FR3T3 cell lines infected with the wild-type ErbB4 retrovirus or the constitutively active ErbB4 mutant retroviruses. However, ErbB4 tyrosine phosphorylation is observed only in the FR3T3 cell lines infected with the constitutively active ErbB4 mutant retroviruses (Fig. 6, *upper panel*). The amount of phosphorylation exhibited by the ErbB4 mutants is less than the amount of phosphorylation exhibited by the constitutively active ErbB2 mutant. Furthermore, the expression of wild-type ErbB4 appears to be less than the expression of the ErbB4 mutants. Nonetheless, these data suggest that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts is not attributable to an absence of expression and tyrosine phosphorylation of these mutants in these cells.

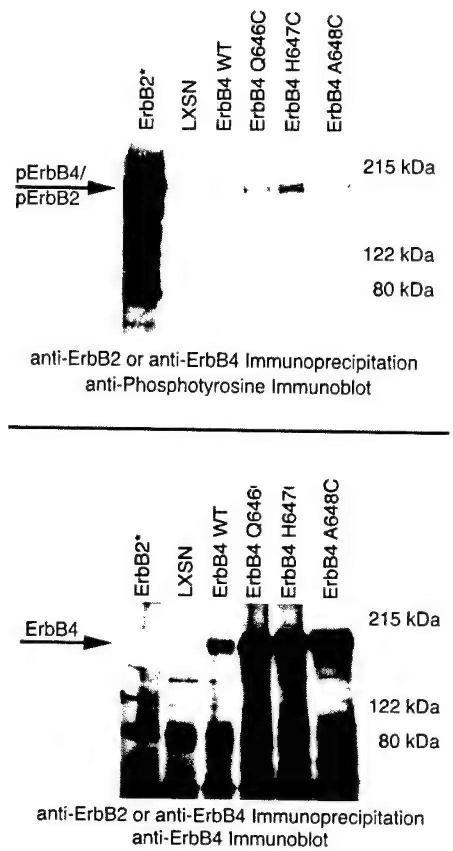

Fig. 6. Constitutively active ErbB4 mutants are expressed and are constitutively tyrosine phosphorylated in FR3T3 cells. ErbB4 expression and tyrosine phosphorylation were assayed in FR3T3 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants. Cells infected with the LXSN recombinant retrovirus vector control or with the ErbB2* retrovirus served as controls. Lysates were prepared from each of the cell lines, and ErbB receptors were precipitated from 1.5 mg of each lysate using protein A-Sepharose and either an anti-ErbB4 rabbit polyclonal antibody or an anti-ErbB2 rabbit polyclonal antibody. Samples were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (*upper panel*). The blot was then stripped and probed with an anti-ErbB4 rabbit polyclonal antibody (*lower panel*). *Arrows,* positions of ErbB2 and ErbB4 on the blots.

Discussion

In this report, we describe the construction and initial characterization of three constitutively active ErbB4 mutants. These mutants display increased dimerization (data not shown) and ligand-independent tyrosine phosphorylation and kinase activity. In these respects, the ErbB4 mutants resemble constitutively active mutants of ErbB2 or EGFR. However, unlike constitutively active ErbB2 mutants, these mutants are not coupled to malignant growth transformation in FR3T3 fibroblasts; they do not induce foci, anchorage-independent growth, or increases in the growth rate or saturation density. These data suggest that ErbB2 and ErbB4 play distinct roles in tumorigenesis *in vivo*. This conclusion is supported by the observation that NIH3T3 clone 7d cells do not form foci after ErbB4 transfection and treatment with the ErbB4 ligand neuregulin but do form foci after ErbB2 and ErbB4 cotransfection and neuregulin treatment (32, 33).

Of course, another potential explanation is that the amounts of tyrosine phosphorylation displayed by the three constitutively active ErbB4 mutants are insufficient to couple to malignant growth transformation in fibroblasts. This is consistent with the observation that the three constitutively active ErbB4 mutants are less phosphorylated than the constitutively active ErbB2 mutant (Fig. 6). However, anti-phosphotyrosine immunoblotting is not a sensitive method for assessing ErbB family receptor signaling and coupling to biological responses. Indeed, the neuregulin concentration required for maximal ErbB4 tyrosine phosphorylation is ~10-fold greater than the neuregulin concentration sufficient for maximal ErbB family receptor coupling to biological responses. Furthermore, the neuregulin concentration sufficient for maximal ErbB family receptor coupling to biological responses stimulates, at most, only modest amounts of ErbB4 tyrosine phosphorylation (26). Thus, it is not likely that the failure of the constitutively active ErbB4 mutants to couple to malignant growth transformation in fibroblasts is attributable to insufficient ErbB4 tyrosine phosphorylation.

Clearly, additional work is necessary to define the roles that ErbB4 plays in tumorigenesis and in regulating cellular functions *in vivo*. However, important clues have emerged to guide these future studies. In a significant percentage of breast tumor samples, ErbB4 expression correlates with estrogen receptor expression, which indicates a favorable prognosis (16–17). Furthermore, ErbB4 expression is fre-

quently lost in tumors of the breast and prostate (19). Finally, ligands for ErbB4 can induce terminal differentiation and growth arrest of some mammary tumor cell lines (35–37). These data indicate that ErbB4 signaling may be coupled to differentiation, growth arrest, and tumor suppression. The ErbB4 mutants described in this study will enable us to evaluate this hypothesis. Indeed, preliminary data from our laboratory indicate that the Q646C ErbB4 mutant causes reduced colony formation in plastic dishes by a number of cultured human breast and prostate tumor cell lines.

We will also perform additional studies to characterize the biochemistry of signaling by the three ErbB4 mutants. Whereas these mutants exhibit greater ligand-independent tyrosine phosphorylation and autokinase activity than the wild-type receptor, it is unclear whether this is attributable to increased intrinsic kinase activity or attributable to increased availability of the substrate. Additional experiments are warranted to distinguish between these two possibilities.

Another area of future study will focus on identifying the mechanisms by which ErbB4 is coupled to biological responses. Initial studies will identify the sites of ErbB4 tyrosine phosphorylation for these mutants. If our preliminary studies indicating that the Q646C ErbB4 mutant is coupled to prostate and mammary tumor cell growth arrest hold true, then we will use genetic strategies to identify the sites of ErbB4 tyrosine phosphorylation that are sufficient and necessary to couple the Q646C ErbB4 mutant to this biological response. A similar strategy has been used to identify the sites of ErbB2 and platelet-derived growth factor receptor tyrosine phosphorylation that are critical for coupling these receptors to biological responses (38, 39).

Once we have identified the site(s) of tyrosine phosphorylation that is sufficient for coupling to biological responses, we will identify signaling proteins that bind this phosphorylation site and couple it to biological responses. Using this strategy, we will begin to characterize the ErbB4 signaling pathway. Our prediction is that the three constitutively active ErbB4 mutants are phosphorylated on different tyrosine residues and that these mutants differentially couple to biological responses. We have shown previously that different ErbB4 ligands cause phosphorylation on different sites on ErbB4 and differential coupling to biological responses (40). Moreover, one cysteine substitution mutation in the rat ErbB2 extracellular domain (V656C) results in low amounts of constitutive receptor tyrosine phosphorylation and efficient coupling to malignant growth transformation in rodent fibroblasts. In contrast, another rat ErbB2 extracellular domain cysteine substitution mutant (T657C) exhibits very high levels of constitutive receptor tyrosine phosphorylation but a relatively low amount of coupling to malignant growth transformation in rodent fibroblasts (28).

We were somewhat surprised to discover that the three constitutively active ErbB4 mutants failed to couple to malignant growth transformation in a rodent fibroblast cell line. Nonetheless, these mutants will enable us to assess ErbB4 function in a wide variety of cell, tissue, and organismal contexts. Given that ErbB4 appears to regulate diverse functions in a number of distinct contexts, much work remains to complete this story.

Materials and Methods

Cell Lines, Cell Culture, and Antibodies. The Ψ2, PA317, C127, and FR3T3 cell lines were generous gifts from Daniel DiMaio (Yale University New Haven, CT). All cell lines were propagated in DMEM supplemented with 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin (Mediatech), and 0.25 μg/ml Fungizone (Amphotericin B; Life Technologies, Inc.). Recombinant cell lines generated in the course of the experiments described in this report were propagated in the medium described above supplemented with 200 μg/ml G418 (Mediatech).

The anti-ErbB4 mouse monoclonal (SC-8050), anti-ErbB4 rabbit polyclonal (SC-283), and anti-ErbB2 rabbit polyclonal (C-18) antibodies were purchased from Santa Cruz Biotechnology. Goat antimouse and goat antirabbit horseradish peroxidase-conjugated antibodies were purchased from Pierce. Enhanced chemiluminescence (ECL) Western blotting reagents, Redivue adenosine 5′-[γ-^{32}P]triphosphate, and Protein-A Sepharose (CL-4B) were purchased from Amersham Pharmacia Biotech. The 4G10 anti-phosphotyrosine mouse monoclonal antibody was purchased from Upstate Biotechnology.

Plasmids. The recombinant retroviral vector pLXSN (41) was obtained from Daniel DiMaio (Yale University). This construct contains two recombinant LTRs derived from the Maloney murine leukemia virus and the Maloney murine sarcoma virus. These LTRs flank the Ψ packaging signal and the aminoglycoside 3′-phosphotransferase (*Neo*^{R}) gene under the transcriptional control of the SV40 early promoter. The *Neo*^{R} gene confers resistance to the aminoglycoside antibiotic G418 (geneticin; Life Technologies, Inc.).

The recombinant retroviral construct pLXSN-ErbB4 (26) was generated by subcloning the human ErbB4 cDNA into pLXSN. In this construct, the ErbB4 cDNA is under the transcriptional control of the upstream LTR. The recombinant retroviral construct pLXSN-ErbB2* (42) was a gift of Lisa Petti (Albany Medical College, Albany, NY). It was generated by subcloning the cDNA encoding the constitutively active rat ErbB2 mutant (V664E transmembrane domain mutant, ErbB2*) into pLXSN. In this construct, the ErbB2* cDNA is under the transcriptional control of the upstream LTR.

ErbB4 Mutagenesis. The plasmid pLXSN-ErbB4 was used as the template for site-directed mutagenesis (QuikChange Site Directed Mutagenesis kit; Stratagene) to construct the putative constitutively active ErbB4 mutants. The mutants were constructed by introducing mutations that substitute a cysteine residue for proline 645, glutamine 646, histidine 647, alanine 648, or arginine 649 in the ErbB4 extracellular juxtamembrane domain. These mutants are denoted as follows: P645C, Q646C, H647C, A648C, and R649C. A new restriction enzyme site was also engineered in each mutant to facilitate the identification of the mutants. The following primers were used for mutagenesis. "T" denotes the upper primer, whereas "B" denotes the lower primer. The novel cysteine codons and anticodons are indicated by bold type, the point mutations that create the novel cysteine residues are double underlined, and the novel restriction enzyme sites are singly underlined.

P645CT:5′- ATTTACTACCCATGGACCGGTCATTCCACTT
TATGCCAACATGCTAGAACTCCC-3′
P645CB:5′- GGGAGTTCTAGCATGTTGGCATAAAGTGGA
ATGACCGGTCCATGGGTAGTAAAT-3′
Q646CT:5′- TACTACCCATGGACCGGTCATTCCACTTTAC
CATGCCATGCTAGAACTCCCCTG-3′
Q646CB:5′-CAGGGGAGTTCTAGCATGGCATGGTAAAGT
GGAATGACCGGTCCATGGGTAGTA-3′
H647CT:5′- CATTTACTACCCATGGACCGGTCATTCCACT
TTACCACAATGTGCTAGAACTCCCCT-3′
H647CB:5′-AGGGGAGTTCTAGCACATTGTGGTAAAGTG
GAATGACCGGTCCATGGGTAGTAAATG-3′
A648CT:5′- TCCACTTTACCACAACATTGTAGAACTCCTC
TGATTGCAGCTGGA-3′
A648CB:5′- TCCAGCTGCAATCAGAGGAGTTCTACAATG
TTGTGGTAAAGTGGA-3′
R649CT:5′- ACTTTACCACAACATGCTTGCACTCCTCTGA
TTGCAGCTGGA-3′
R649CB:5′-TCCAGCTGCAATCAGAGGAGTGCAAGCATG
TTGTGGTAAAGT-3′

The site-directed mutagenesis reactions were performed according to the manufacturer's instructions. Standard techniques (43) were used for bacterial transformations, small-scale plasmid DNA preparations, restriction enzyme analysis of the clones, and large-scale plasmid DNA preparations. Positive clones were sequenced by the University of Wisconsin-Madison Biotechnology Center to confirm their identity.

Production of Recombinant Retroviruses and Retroviral Infections. The ErbB4 mutant constructs were transfected using standard techniques (44, 45) into the $\psi 2$ ecotropic retrovirus packaging cell line (46) to generate cell lines that express the ErbB4 mutants and to package the constructs into low-titer ecotropic retrovirus particles (44, 45). $\psi 2$ cells were transfected with the pLXSN vector control plasmid, pLXSN-ErbB4, and pLXSN-ErbB2* to generate control cell lines and recombinant ecotropic retroviruses. The PA317 amphotropic packaging cell line (47) and the FR3T3 rat fibroblast cell line were infected with the ecotropic recombinant retroviruses using standard techniques (44, 45) to generate additional cell lines that express the ErbB4 mutants.

Immunoblot Assays for Receptor Tyrosine Phosphorylation and Expression. The analysis of ErbB4 and ErbB2 tyrosine phosphorylation by immunoprecipitation and anti-phosphotyrosine immunoblotting has been described previously (21, 26). Briefly, cell lysates were generated, and protein content was quantified using a Coomassie Protein Assay Reagent (Ref. 48; Pierce Chemical). ErbB2 or ErbB4 was immunoprecipitated from equal amounts of protein using specific antibodies. The immunoprecipitates were resolved by SDS-PAGE on a 7.5% acrylamide gel and were electrotransferred onto nitrocellulose. The blots were probed with the anti-phosphotyrosine monoclonal antibody 4G10. Antibody binding was detected and visualized using a goat antimouse horseradish peroxidase-coupled antibody and enhanced chemiluminescence. The blots were then stripped and probed with the anti-ErbB4 polyclonal antibody to assess ErbB4 expression levels. Antibody binding was detected and visualized using a goat antimouse horseradish peroxidase-coupled antibody and enhanced chemiluminescence.

The amounts of receptor tyrosine phosphorylation and expression were quantified by digitizing the chemilumigrams using a Linotype-Hell Jade two-dimensional scanning densitometer set at 600-dpi resolution. The bands on the images were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net receptor expression and tyrosine phosphorylation values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

***In Vitro* Kinase Assay.** ErbB2 and ErbB4 were immunoprecipitated from protein extracts from PA317 cells as described previously (26). Immune complex kinase reactions were performed as described previously (31). Briefly, 35 μl of protein A-Sepharose and 5 μl of anti-ErbB2 or anti-ErbB4 rabbit polyclonal antibodies were used to immunoprecipitate the receptors from lysates containing the same amount of protein (1000 μg). Immunoprecipitates were washed five times in 500 μl of kinase buffer [20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 3 mM $MnCl_2$]. After the last wash, the samples were resuspended in 100 μl of kinase buffer supplemented with 10 μCi of [γ-^{32}P]ATP and were incubated for 10 min at room temperature to permit the kinase reaction to occur. The beads were then washed two times in NET-N buffer (49) and boiled for 5 min in SDS-PAGE protein sample buffer. The samples were resolved by SDS-PAGE on a 7.5% acrylamide gel. The gels were dried overnight and exposed to X-ray film for ~20 h. The autoradiograms were digitized using a Linotype-Hell Jade two-dimensional scanning densitometer set at 600-dpi resolution. The bands on the images were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net kinase activity values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

Focus Formation Assay for Loss of Contact Inhibition. FR3T3 and C127 cells were infected with recombinant ecotropic retroviruses as described earlier and in reports published previously (44, 45). Briefly, 60-mm dishes of cells at ~70% confluence were infected with ecotropic retrovirus stocks. Approximately 24 h after infection, cells were passaged into three 60-mm dishes. Cells were maintained in DMEM supplemented with 10% FBS until foci appeared. During this period, the medium was changed every 3 days. Once robust foci appeared, cells were fixed in 100% methanol and stained with Giemsa (Fisher) to visualize the foci. The plates were digitized using a Linotype-Hell Jade two-dimensional scanning densitometer set at 600-dpi resolution. The digitized images were cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

Assay for Anchorage Independence. FR3T3 cells were seeded at a density of 2 × 10^4 cells in 60-mm dishes containing 2.5 ml of 0.3% LMP-agarose (Life Technologies, Inc.)

as described previously (50). Every 3 days, DMEM supplemented with 10% FBS and 0.3% LMP-agarose was added to each plate. The cells were incubated at 37°C for 10 days, and fields were photographed with an Olympus OM-10 camera attached to an Olympus CK-2 phase-contrast inverted microscope. The images were digitized by the photofinisher. These images were cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software. Images are representative of three independent experiments.

Growth Rate/Saturation Density Assay. Stable FR3T3 cell lines expressing the wild-type ErbB4 receptor, ErbB2*, or the ErbB4 mutants (Q646C, H647C, and A648C) were plated in 10 60-mm dishes at a density of 2×10^4 cells/dish. Cells were incubated from 1 to 10 days at 37°C. Cells were counted (Coulter Counter ZM) each day for a total of 10 days. The mean and SE are representative of three independent experiments.

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A constitutively active ErbB4 mutant inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines

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Abstract

ErbB4 (HER4) is a member of the ErbB family of receptor tyrosine kinases, a family that also includes the Epidermal Growth Factor Receptor (EGFR/ErbB1/HER1), Neu/ErbB2/HER2, and ErbB3/HER3. Several groups have hypothesized that signal transduction by the ErbB4 receptor tyrosine kinase is coupled to differentiation, growth arrest, and tumor suppression in mammary and prostate epithelial cells. In this report we demonstrate that a constitutively active ErbB4 mutant inhibits the formation of drug-resistant colonies by the DU-145 and PC-3 human prostate tumor cell lines. This is consistent with our hypothesis that ErbB4 signaling is growth inhibitory and may be coupled to tumor suppression in prostate cells.

Keywords: ErbB4; Receptor tyrosine kinase; Growth inhibition; Tumor suppression; Prostate cancer

1. Introduction

ErbB4 is a member of the ErbB family of receptor tyrosine kinases, a family that also includes the epidermal growth factor (EGF) receptor (EGFR/ErbB1/HER1), ErbB2/HER2/Neu, and ErbB3/HER3 [1–3]. The agonists for these receptors are members of the EGF family of peptide hormones, which includes more than 20 different growth factors (reviewed in [2–4]. The signaling network comprised of these hormones and receptors regulates cell proliferation and differentiation, as well as other cellular functions. Moreover, deregulated signaling by this network, typically due to inappropriate receptor or ligand (over)expression, plays a significant role in many human tumors [3,5–7]. For example, EGFR or ErbB2 overexpression is detected in a significant percentage of human breast tumors and this overexpression correlates with increased metastatic potential, chemoresistance, and poorer patient prognosis.

In contrast, relatively little is known about the roles that ErbB4 plays in tumorigenesis. ErbB4 overexpression is much less common in mammary tumor samples than is EGFR or ErbB2 overexpression. Moreover, ErbB4 overexpression in mammary tumor samples correlates with a more favorable prognosis,

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not a less favorable prognosis [8–11]. The expression of ErbB4 and its ligands in the developing mouse mammary epithelium is highest late in pregnancy and during lactation, and corresponds with a period of terminal differentiation of the mammary epithelium and only limited proliferation [12,13]. Finally, the normal human prostate epithelium exhibits abundant ErbB4 expression; in contrast, ErbB4 expression has not been detected in any cultured human prostate tumor cell line studied to date [14,15]. These data have led investigators to hypothesize that ErbB4 signaling is coupled to terminal differentiation, growth arrest, and tumor suppression in the mammary and prostate epithelia.

A typical strategy for studying the function of a given ErbB family receptor involves assessing the effect of an EGF family hormone that binds to the ErbB family receptor of interest. These studies can be done either in cells that endogenously express the receptor of interest or in cells that overexpress the appropriate receptor. However, EGF family hormones stimulate heterodimerization of the cognate (binding) ErbB family receptor with any other ErbB family receptor present. This results in tyrosine phosphorylation and signaling by both the cognate ErbB family receptor as well as any other ErbB receptor. Thus, in human breast and prostate tumor cell lines, which frequently express EGFR, ErbB2, and ErbB3, ligands for ErbB4 stimulate not only ErbB4 signaling, but signaling by the other ErbB family receptors as well. Consequently, stimulation with ErbB4 ligands has been of limited value in studying ErbB4 function. Nonetheless, the ErbB4 ligand Neuregulin1beta (NRG1β) stimulates differentiation of mammary epithelium to lobuloalveoli in vivo [16] and stimulates in vitro differentiation of the AU-565 human tumor cell line [17,18]. Furthermore, ErbB4 expression in the SUM102 human mammary tumor cell lines permits the induction of differentiation and growth inhibition by NRG1β [19]. However, efforts by our laboratory to extend these results to other human breast tumor cell lines and to prostate tumor cell lines have failed.

In response, we have embarked on a genetic strategy to study ErbB4 function. We have previously reported the construction of three constitutively active human ErbB4 mutants. These mutants are the result of a single cysteine substitution for Gln646, His647, or Ala648 of the ErbB4 extracellular, juxtamembrane domain. Our initial analyses of these mutants revealed that these mutants, unlike a constitutively active ErbB2 mutant, fail to malignantly transform the growth of rodent fibroblast cell lines [20]. In this report we show that one of these mutants inhibits drug-resistant colony formation by two human prostate tumor cell lines. These data suggest that ErbB4 may indeed be coupled to differentiation, growth arrest, and tumor suppression in the prostate epithelium.

2. Materials and methods

2.1. Cell lines and cell culture

Mouse C127 fibroblasts and the ψ2 and PA317 recombinant retrovirus packaging cell lines are generous gifts of Dr Daniel DiMaio (Yale University, New Haven, Connecticut, USA). These cells were cultured essentially as described previously [21,22]. PC-3 and DU-145 human prostate tumor cell lines were obtained from American Type Culture Collection and were cultured in accordance with vendor recommendations. Cell culture media and supplements were obtained from GIBCO/BRL/Life Technologies. Fetal bovine serum and G418 were obtained from Gemini Bioproducts. Plasticware and Giemsa stain were obtained from Fisher Scientific.

2.2. Retrovirus infections and drug-resistant colony formation assays

Recombinant amphotropic retroviruses were produced essentially as described earlier [22]. Briefly, the recombinant retroviral constructs pLXSN (vector) [23], pLXSN-ErbB4 (ErbB4 WT) [24], pLXSN-ErbB2 V664E (ErbB2*) [25], pLXSN-ErbB4 Q646C, pLXSN-ErbB4 H647C, and pLXSN-ErbB4 A648C [20] were transfected into the ψ2 ecotropic retrovirus packaging cell line [26]. Transfected cells were selected using G418 and drug-resistant colonies were pooled and expanded into stable cell lines. Recombinant ecotropic retroviruses were recovered from the conditioned media of the recombinant ψ2 cell lines. These stocks were used to infect the PA317 amphotropic retrovirus packaging cell line [27].

Infected cells were selected using G418 and drug-resistant colonies were pooled and expanded into stable cell lines. Recombinant amphotropic retroviruses were recovered from the conditioned media of the recombinant PA317 cell lines. pLXSN is a generous gift of Dr Daniel DiMaio (Yale University, New Haven, Connecticut, USA). pLXSN-ErbB2* is a generous gift of Dr Lisa Petti (Albany Medical College, Albany, New York, USA).

C127, DU-145, and PC-3 infections with the recombinant amphotropic retroviruses were performed essentially as described earlier [20–22]. Infected cells were selected using G418. Approximately 12 days after infection, drug-resistant colonies were stained using Giemsa. The tissue culture plates were digitized using an Epson flatbed scanner set for 600 dpi. The digital images were cropped, annotated and combined into composite images. The contrast of the images was enhanced and the background was minimized to maximize the signal–noise ratio. Manipulations of the digital images were performed using Adobe Photoshop.

Drug-resistant colonies were counted manually and the retrovirus titer for each combination of retrovirus and cell line was determined by dividing the number of colonies by the volume of retrovirus used in the infection. The average viral titers were calculated from at least ten independent sets of infections. The efficiency of drug-resistant colony formation was calculated for each retrovirus stock in the DU-145 cell line by dividing the retroviral titers in the DU-145 cells by the corresponding retroviral titers in the C127 cells. These values are expressed as mean percentages calculated from at least ten independent sets of infections. The standard error was also calculated for each mean percentage. Analogous calculations were performed to calculate the efficiency of drug-resistant colony formation for each retrovirus stock in the PC-3 cell lines.

2.3. Immunoprecipitation and immunoblotting

Anti-ErbB4 immunoprecipitations and anti-phosphotyrosine immunoblotting were performed essentially as described earlier [20]. Briefly, C127 cells were starved overnight in serum-free medium, then lysed using an ice-cold isotonic lysis buffer supplemented with the non-ionic detergent NP-40 (Sigma). Nuclei and cellular debris were cleared from the lysates by centrifugation. The protein content of the lysate supernatants was determined using a modified Bradford protein assay (Pierce). ErbB4 was immunoprecipitated from equal amounts of lysate using protein A sepharose (Amersham/Pharmacia) and an anti-ErbB4 rabbit polyclonal antibody (Santa Cruz Biotechnology). The precipitates were washed with an isotonic lysis buffer and the proteins were released from the sepharose beads by boiling in a reducing SDS sample buffer. The samples were resolved by SDS-PAGE using a 7.5% acrylamide gel and were electroblotted onto nitrocellulose. The resulting blot was probed with an anti-phosphotyrosine mouse monoclonal antibody (Upstate Biotechnology). Primary antibody binding was detected and visualized using a goat anti-mouse antibody conjugated to horseradish peroxidase (Pierce) and enhanced chemiluminescence (Amersham/Pharmacia). The chemilumigram was digitized using an Epson flatbed scanner set for 600 dpi resolution. The digital images were cropped and annotated using Adobe Photoshop.

3. Results

3.1. The ErbB4 Q646C mutant inhibits drug-resistant colony formation by the DU-145 human prostate tumor cell line

We previously described the construction and packaging of recombinant retroviral vectors that express the neomycin resistance gene as well as the constitutively active ErbB4 mutants [20]. We infected DU-145 cells with these retroviruses and selected for drug-resistant colonies using G418 to assess whether any of the constitutively active ErbB4 mutants inhibits drug-resistant colony formation. As controls we also infected DU-145 cells with recombinant retroviruses that carry only the neomycin resistance gene (Vector), with recombinant retroviruses that express a constitutively active (V664E) mutant of the rat ErbB2 gene (ErbB2*) [25], and with recombinant retroviruses that express the wild-type ErbB4 gene. To control for differences in absolute viral titers, we infected C127 mouse fibroblasts in parallel and

assayed the formation of drug-resistant colonies of infected cells.

As shown in Fig. 1, DU-145 cells infected with the recombinant retrovirus that expresses the ErbB4 Q646C mutant form fewer drug-resistant colonies than do DU-145 cells infected with the other recombinant retroviruses. Furthermore, the titer of the ErbB4 Q646C recombinant retrovirus in the DU-145 cells is less than the titers of the other recombinant retroviruses (Table 1). However, the titer of the ErbB4 Q646C recombinant retrovirus in C127 fibroblasts is not less than the titer of most of the other recombinant retroviruses (Table 1). Thus, the ratio of the ErbB4 Q646C retroviral titers in DU-145 and C127 cells is much less than the corresponding ratios of the other retrovirus titers (Table 1). Indeed, it appears that the ErbB4 Q646C mutant inhibits drug-resistant colony formation by DU-145 cells by approximately 90%.

3.2. The ErbB4 Q646C mutant inhibits drug-resistant colony formation by the PC-3 human prostate tumor cell line

We infected PC-3 cells in parallel with the DU-145 and C127 infections. The results of these infections

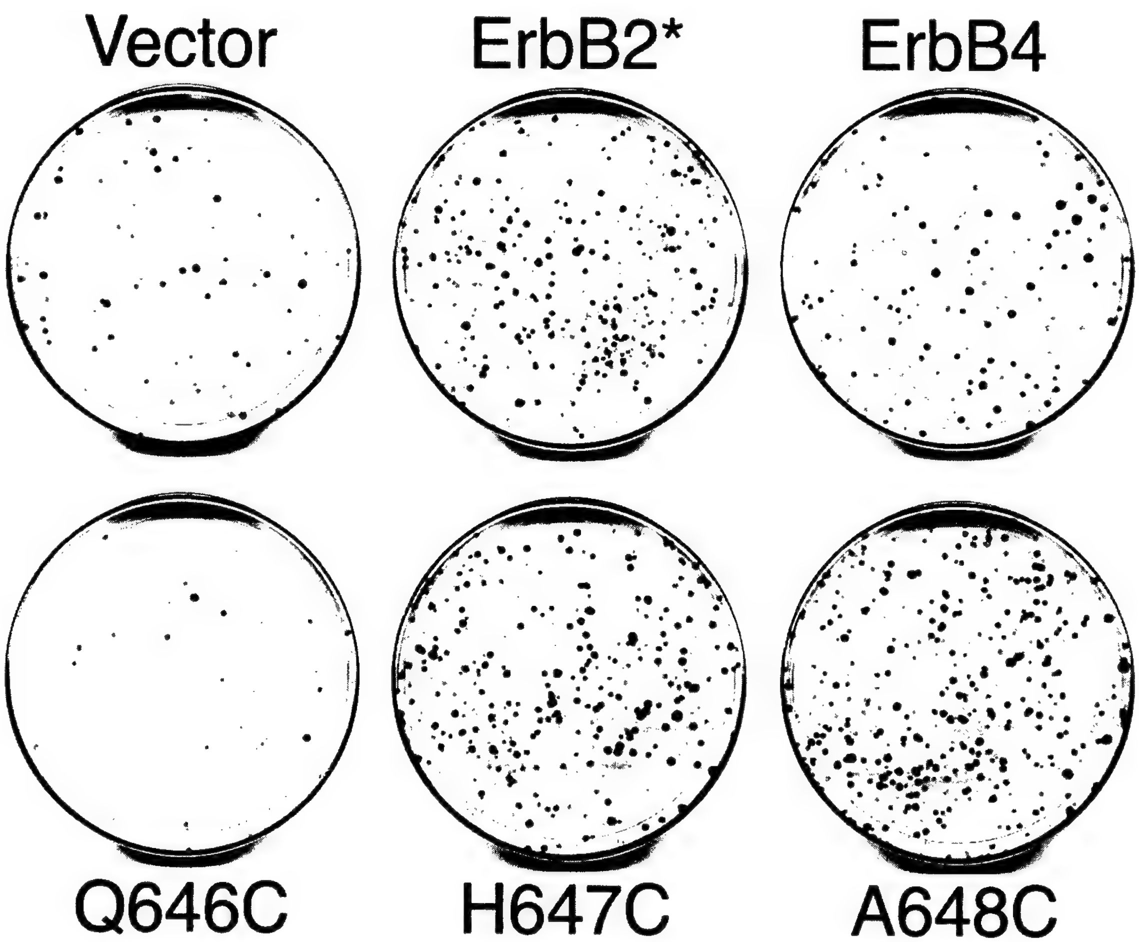

Fig. 1. The ErbB4 Q646C mutant inhibits drug-resistant colony formation by the DU-145 human prostate tumor cell line. DU-145 human prostate cells were infected with recombinant amphotropic retroviruses that carry the neomycin resistance gene (Vector) or with retroviruses that carry the neomycin resistance gene along with a constitutively active ErbB2 mutant (ErbB2*), wild-type ErbB4 (ErbB4), or constitutively active ErbB4 mutants (Q646C, H647C, A648C). Infected cells were selected using 600 μg/ml G418. Colonies of drug-resistant cells were stained using Giemsa and counted.

Table 1
The ErbB4 Q646C mutant specifically inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines[a]

	Viral titers			Colony formation efficiency	
Virus	Cell line			Ratios	
Stock	C127	DU-145	PC-3	DU-145/C127	PC-3/C127
Vector	1.14E + 06	7.88E + 04	1.21 E + 05	10.7 ± 2.7	19.4 ± 5.7
ErbB2*	2.92E + 05	3.23E + 04	3.09E + 04	11.9 ± 1.8	15.6 ± 3.9
ErbB4 WT	1.55E + 05	1.44E + 04	2.27E + 04	12.0 ± 3.1	25.3 ± 7.5
Q646C	6.17E + 05	3.42E + 03	1.56E + 04	0.6 ± 0.1	3.1 ± 0.8
H647C	8.65E + 05	4.59E + 04	6.27E + 04	7.2 ± 1.2	17.3 ± 6.3
A648C	1.49E + 05	1.46E + 04	1.67E + 04	11.8 ± 2.1	15.0 ± 2.8

[a] We counted the number of colonies on each plate of infected DU-145, PC-3, and C127 cells and divided by the volume of retrovirus used to infect the cells to determine the titer of each retrovirus stock in each of the three cell lines. To compare the relative efficiency of each retrovirus stock at inducing drug-resistant colony formation in the DU-145 cell line, we divided the titer of each retrovirus stock in the DU-145 cell line by the titer of the same retrovirus stock in the C127 cell line. This value is expressed as a mean percentage calculated from at least ten independent sets of infections. The standard error for each mean was calculated and is reported. We performed analogous calculations to determine the efficiency of drug-resistant colony formation of each retrovirus stock in the PC-3 cell lines.

are similar to the results of the DU-145 infections. PC-3 cells infected with the recombinant retrovirus that expresses the ErbB4 Q646C mutant form fewer drug-resistant colonies than do PC-3 cells infected with the recombinant retroviruses that express the other ErbB4 constructs (Fig. 2). Furthermore, the titer of the ErbB4 Q646C recombinant retrovirus in the PC-3 cells is less than the titers of the other recombinant retroviruses (Table 1). Finally, the ratio of the ErbB4 Q646C retroviral titers in PC-3 and C127 cells is much less than the corresponding ratios of the other retrovirus titers (Table 1). Indeed, it appears that the ErbB4 Q646C mutant inhibits drug-resistant colony formation by PC-3 cells by approximately 75%.

3.3. The constitutively active ErbB4 mutants are expressed and tyrosine phosphorylated in the mouse C127 fibroblast cell line

We were concerned that the failure of the ErbB4 H647C and A648C mutants to inhibit drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines may be due to an absence of expression and/or tyrosine phosphorylation of these ErbB4 mutants. Consequently, we pooled drug-resistant colonies that resulted from infections of C127 cells and generated stable cell lines. We assayed ErbB4 expression and tyrosine phosphorylation in these cell lines by ErbB4 immunoprecipitation and anti-phosphotyrosine immunoblotting.

In Fig. 3 we show that all three constitutively active ErbB4 mutants are expressed and display ligand-independent tyrosine phosphorylation in the appropriate C127 cell lines. Indeed, it appears that the ErbB4 Q646C mutant exhibits less tyrosine phosphorylation than the ErbB4 H647C and A648C mutants. This suggests that the failure of the ErbB4 H647C and A648C mutants to inhibit drug-resistant colony formation by the DU-145 and PC-3 cell lines is not due to an absence of expression and/or tyrosine phosphorylation of these ErbB4 mutants.

4. Discussion

Here we demonstrate that the Q646C constitutively active ErbB4 mutant inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines. This suggests that ErbB4 signaling is coupled to prostate cell growth arrest and tumor suppression. Several issues remain to be resolved in future experiments.

The phenotype that underlies ErbB4 coupling to inhibition of drug resistant colony formation has yet to be determined. For example, it is possible that ErbB4 couples to specific cell cycle arrest. However, it is also possible that ErbB4 is coupling to apoptosis rather than

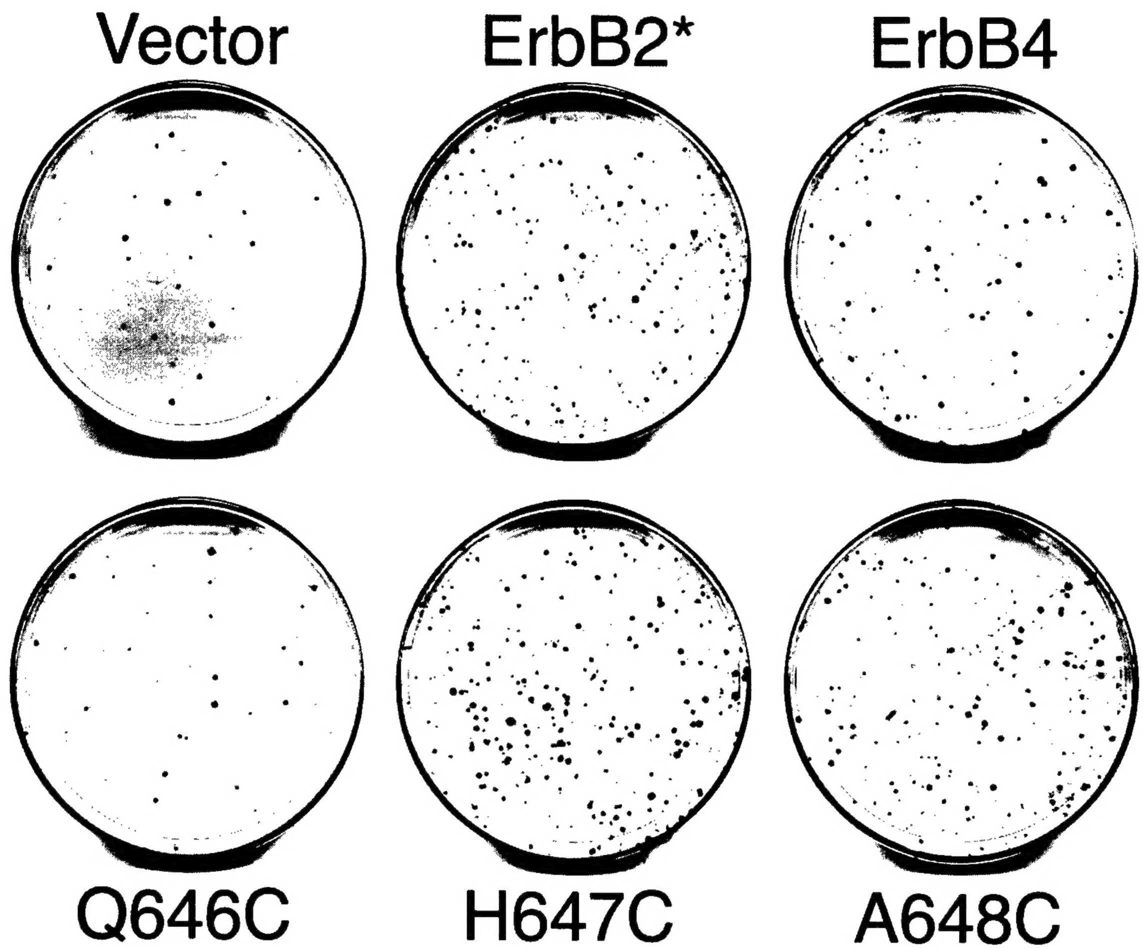

Fig. 2. The ErbB4 Q646C mutant inhibits drug-resistant colony formation by the PC-3 human prostate tumor cell line. PC-3 human prostate cells were infected with recombinant amphotropic retroviruses that carry the neomycin resistance gene (Vector) or with retroviruses that carry the neomycin resistance gene along with a constitutively active ErbB2 mutant (ErbB2*), wild-type ErbB4 (ErbB4), or constitutively active ErbB4 mutants (Q646C, H647C, A648C). Infected cells were selected using 600 μg/ml G418. Colonies of drug-resistant cells were stained using Giemsa and counted.

growth arrest. Since it is impossible to evaluate these hypotheses with the experimental system described in this report, we are developing a conditional expression system that should enable us to determine whether ErbB4 signaling is coupled to cell cycle arrest, apoptosis, or non-specific growth arrest.

Another goal for future experiments is to determine why the Q646C ErbB4 mutant is coupled to inhibition of drug-resistant colony formation by prostate tumor cell lines, whereas the H647C and A648C ErbB4 mutants are not. The differential coupling of these ErbB4 mutants is analogous to the differential coupling of constitutively phosphorylated rat ErbB2 mutants to growth transformation of rodent fibroblasts [28]. It is also analogous to the differential coupling of mutants of the bovine papillomavirus (BPV) E5 protein to malignant growth transformation of rodent fibroblasts. This differential coupling is in marked contrast to the fact that several of these BPV E5 mutants stimulate abundant platelet-derived growth factor receptor tyrosine phosphorylation [29,30]. In both of these examples, it is believed that the constitutively phosphorylated receptor tyrosine kinases are phosphorylated on different individual tyrosine residues, resulting in differential coupling to downstream signaling proteins and biological

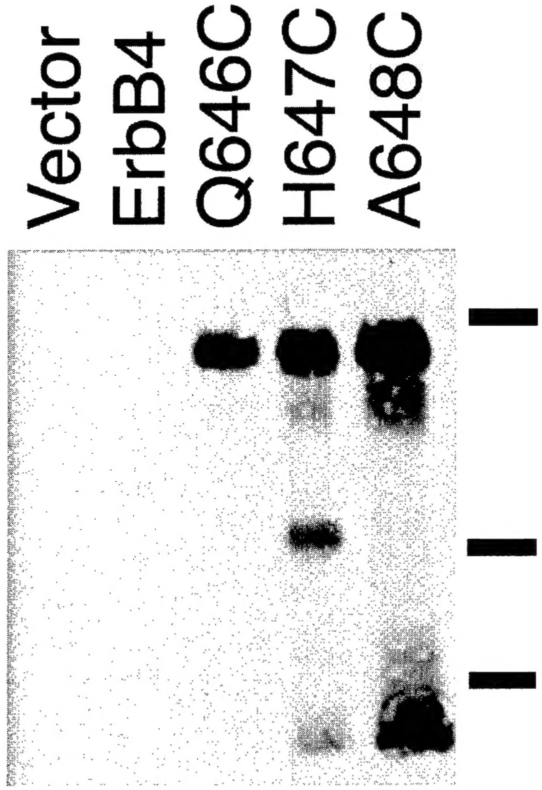

Fig. 3. The constitutively active ErbB4 mutants are expressed and tyrosine phosphorylated in the mouse C127 fibroblast cell line. C127 fibroblasts were infected with recombinant amphotropic retroviruses that express the neomycin resistance gene (Vector) or with retroviruses that express the neomycin resistance gene along with wild-type ErbB4 (ErbB4) or constitutively active ErbB4 mutants (Q646C, H647C, A648C). Infected cells were selected using 1000 μg/ml G418. Colonies of drug-resistant cells were pooled and expanded into stable cell lines. Confluent 100 mm plates of each cell line were incubated in serum-free medium for 24 h, after which cells were lysed. ErbB4 was precipitated using specific antibodies and the precipitates were resolved by SDS-PAGE and electroblotted onto nitrocellulose. The blot was probed with an anti-phosphotyrosine mouse monoclonal antibody. Antibody binding was detected and visualized using a goat anti-mouse secondary antibody coupled to horseradish peroxidase and enhanced chemiluminescence. Bars indicate the positions of the molecular weight markers (198 kDa, 115 kDa, and 93 kDa). Tyrosine phosphorylated ErbB4 is represented by the band with apparent mobility of approximately 190 kDa.

responses. Indeed, different ErbB4 ligands cause different patterns of ErbB4 phosphorylation and differential coupling to downstream signaling effectors and biological responses [31]. Thus, we hypothesize that the functional differences between the ErbB4 Q646C mutant and the other constitutively active ErbB4 mutants are due to phosphorylation on different ErbB4 tyrosine residues. Mapping the sites of ErbB4 tyrosine phosphorylation for the three constitutively active ErbB4 mutants and genetic studies to identify the sites of ErbB4 tyrosine phosphorylation that couple ErbB4 to inhibition of drug resistant colony formation will enable us to formally address this hypothesis.

Finally, additional experiments will be necessary to formally test the hypothesis that ErbB4 is a prostate tumor suppressor. Male transgenic mice that exhibit tissue specific ectopic expression of the Q646C ErbB4 mutant in the prostate gland would be an appropriate in vivo model system for assessing whether constitutive ErbB4 signaling is sufficient to suppress prostate tumorigenesis.

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